TRAUMATIZED MUSCLE-DERIVED MULTIPOTENT PROGENITOR CELLS: PRO-ANGIOGENIC ACTIVITY, PROMOTION OF NERVE GROWTH, AND OSTEOGENIC DIFFERENTIATION

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Following trauma from high-energy blasts, a population of multipotent progenitor cells (MPCs) can be derived from the debrided muscle tissue. Given the non-intrusive nature of MPC isolation, MPCs represent an attractive clinical alternative to the more widely used mesenchymal stem cells derived from bone marrow and other tissue sources. This dissertation presents work which further defines the utility and limitations of MPCs in applications relevant to the repair of extremity injuries, including angiogenesis, peripheral nerve repair, and bone formation.

The secretome of MPCs enhanced *in vitro* angiogenesis, in a manner dependent on MPC production of vascular endothelial growth factor-A (VEGF). Encapsulated within mechanically tunable injectable hydrogel constructs, MPCs retained strong pro-angiogenic activity when implanted *in vivo*, supporting potential clinical use when enhanced vessel recruitment is desired.

Neurotrophically-induced MPCs, in combination with endothelial cells (ECs) co-cultured on aligned, nanofibrous scaffolds or via secretome interactions, supported neurite outgrowth and extension of chick embryonic dorsal root ganglia. These findings suggest that products of induced MPCs may be useful to enhance nerve guide conduit-based repair of peripheral nerves.

ECs influenced earlier and stronger MPC osteogenic gene expression, and IL-1β was associated with increased mineralization. The use of MPCs in bone replacement applications may thus result in mineralization without functional bone formation, depending on the level of inflammation at the site of construct implantation.

Taken together, this work extends the potential utility of MPCs for limb regenerative applications, especially for enhanced vessel or nerve recruitment. Caution must be exercised as MPCs may be influenced towards a mineralizing phenotype by the tissue environment, likely contributing to the heterotopic ossification pathology commonly seen following blast trauma.

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LIST OF ABBREVIATIONS

Abbreviation	Full term
AD-MSC	adipose-derived mesenchymal stem cells
ANGPT	angiopoietin
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenetic protein
BSP-II	bone siaoloprotein II
CAM	chorioallantoic membrane assay
CCL	chemokine C-C ligand
CD	cluster of differentiation
CM	conditioned medium (generated under cell-free control or cell culture conditions)
CM+I	conditioned medium, generated in the presence of IL-1β
CNTF	ciliary neurotrophic factor
CRP	c-reactive protein
CXCL	chemokine C-X-C ligand
DMEM	Dulbecco's modified Eagle's Medium
DRG	dorsal root ganglia
EC	microvascular endothelial cells
EC-CM	EC-conditioned medium
EC-CM+I	EC-conditioned medium, generated in the presence of IL-1β
ECM	extracellular matrix
EGF	epidermal growth factor
EPC	endothelial cell progenitor
FBS	fetal bovine serum
FGF-2	basic fibroblast growth factor
FOP	fibrodysplasia ossificans progressiva
GDNF	glial cell line-derived neurotrophic factor
GM-EC	ECs cultured under EC-growth conditions
GM-MPC	MPCs cultured under growth conditions
HBSS	Hank's buffered salt solution
HFF	human foreskin fibroblasts
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
HLA	human leukocyte antigen
HO	heterotopic ossification
IFN	interferon
IGF	insulin-like growth factor
IGFR	IGF receptor
IL	interleukin

Abbreviation	Full term
IRB	Institutional Review Board
ITSX	insulin-transferrin-selenium-X
KGF	keratinocyte growth factor
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MPC-CM	MPC- conditioned medium
MSC	bone marrow-derived mesenchymal stem cells
MSC-CM	MSC- conditioned medium
NEFH	heavy neurofilament
NFC	aligned nanofiber constructs
NFκB	nuclear factor κ-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NM-MPC	MPCs cultured under neurotrophic conditions
NO	nitric oxide
NRP	neuropilin
NT	neurotrophin
OCN	osteocalcin
OEF	Operation Enduring Freedom
OIF	Operation Iraqi Freedom
PBS	phosphate-buffered saline
PPAR	peroxisome proliferator-activated receptor
PS	penicillin-streptomycin
PSF	penicillin-streptomycin- fungizone; antibiotic-antimycotic
PDGF	platelet-derived growth factor
NPP	p-nitrophenyl phosphate substrate
PCL	poly-caprolactone
PGA	poly-glycolic acid
PLA	poly-lactic acid
PU	poly-urethane
PVA	poly-vinyl alcohol
RA	retinoic acid
RUNX2	runt-related transcription factor-2
siRNA	silencing RNA
SDF	stromal-derived factor
TCP	tissue culture-treated plastic
TIMP	tissue inhibitor of metalloproteinase
TNF	tissue necrosis factor
TGF	transforming growth factor
TGF	transforming growth factor
US	United States

Abbreviation	Full term
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
α-MEM	α-minimum essential medium
β-ME	B-mercaptoethanol

PREFACE

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1.0 INTRODUCTION

Recovery following injury on the modern battlefield presents specific challenges. The volume of tissue loss combined with the unique injury mechanism and resulting disorganized wound make the restoration of function an admirable if extremely difficult goal. Before therapies can be developed, the unique role of autologous multipotent progenitor cells in both healing and pathologies must be examined.

1.1 MODERN COMBAT CASUALTIES

Because of recent advances in body armor technology, tourniquet use, improved time-to-treatment, and rapid evacuation to well-equipped hospitals, military combatants are surviving a greater number of more intense combat-related injuries[1]. Improvements in thoracic-protection equate to a reciprocal increase in severity of extremity- and head/brain-trauma, with greater involvement of multiple organ systems in almost all combat-related injuries when compared to previous armed conflicts[2]. A majority (>50%) of combat-related wounds involve the extremities[1].

In contrast with traumatic injuries of the limb in the American civilian population, combat wounds leave very little tissue to stabilize and repair[2]. The volume and extent of tissue lost during combat and subsequent surgical interventions suggests that regeneration and replacement are more desirable goals; to date, these strategies have attempted to exploit the same

mechanisms observed in tissue repair. Adult stem cells have generated intense interest among military researchers due to the young and otherwise healthy nature of most military patients, factors that correlate with stem cell efficacy[3]; the mean age of combat injury for Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) was 23.3 years[4].

1.1.1 Immediate combat casualty care

During 2001-2010, an average of 4000 American military combatants per year were non-mortally wounded in-theater during OIF and OEF[4]. Wound treatment within-theater focused on practical patient stabilization; modern military medics were trained to first address bleeding, through the use of advanced tourniquets and hemostatic dressings, followed by minimal airway and management only if the patient was unconscious[1]. In the absence of hostile fire or adverse weather, helicopter transport allowed removal within one hour to battalion aid stations, equipped with a blood bank and at least four surgeons, or to better-equipped combat support hospitals. Field dressings were removed and patient injuries treated surgically. Depending on prognosis, patients were then evacuated to a Regional Hospital in Germany or, ultimately, to United States (US) military medical centers[1]; transport duration from theater to US hospital ranged from 36 hours to 1 week[5]. Commonly-affected systems and combat-specific complications are described in Table 1[6].

Table 1: Multiple systems may be injured during modern military combat operations[6]

Affected system	Combat-specific complications
Vision	Photoreceptor and retinal neuron loss
Auditory	Sensory hair loss, vertigo
Vasculo-pulmonary/cardiac	Embolism, hemorrhage, or contusion
Central nervous: Brain/spinal cord	Physical and psychological devastation
Peripheral nervous	Limited function, neuropathic pain
Skin graft	Lack of coverage/scar formation
Musculoskeletal	Limited mobility, pain, heterotopic ossification

While solutions to repair each system are being actively investigated, blast extremity injuries typically involve damage to several of these systems, necessitating surgical interventions aimed at ensuring patient survival and limb retention over limb repair.

1.1.2 Follow-up extremity care: limb salvage versus amputation and MPC-isolation

As part of successful wound and infection management, a point of concern in its own right as recalcitrant bacterial and fungal infections cause more than 25% of injured combatants to face revision surgery[7], foreign contaminants that could be identified were removed from blast-damaged tissue through a process of vigorous surgical debridement, termed wound excision[8]. During this procedure, all mangled and 2-3 mm of healthy tissue were excised from the injured tissue in an effort to promote healing and decrease the rate of infection[9], and the area was washed with copious amounts of (often) pressurized sterile saline; this treatment was repeated until the wound closed. From the healthy wound margins, plastic-adherent traumatized-muscle-derived multipotent progenitor cells (MPCs) could be isolated; they will be discussed in further detail in subsequent sections.

Despite these prophylactic interventions, approximately 140 military patients per year (about 3.5%) required lower-limb amputations due to combat-related wounds[10]. Similar to the civilian population, speedy resolution to the question of amputation or limb salvage was key to a positive patient outcome; attempted limb salvage followed by amputation (>90 days after injury) was associated with higher rates of psychological as well as physical problems, including infection and pain compounded by PTSD and substance abuse[11]. Clearly, more effective limb salvage techniques are needed.

1.1.3 Extremity complications

A recent study of military personnel who underwent amputation versus limb salvage showed that amputees typically recovered more musculoskeletal function than limb salvage patients, possibly due to advances in prostheses and early, focused rehabilitation[12]. Orthopedic complications account for the majority of long-term military disabilities[13]; this indicates that vast improvements to limb salvage techniques are required, particularly focusing on functional restoration of the limb.

In a thorough discussion focusing on extremity-related consequences of combat injury, a military panel identified six broad types of complications that required revision surgery; three topics described musculoskeletal complications with two, posttraumatic osteoarthritis and heterotopic ossification (HO), involving excessive and accelerated formation of bone. Another section on complex limb injuries described the need to support vascularization and encourage nerve regeneration, both in the context of limb salvage and transplant support[14]. Twenty-five percent of injured Vietnam veterans suffered peripheral nerve complications, and the incidence from recent conflicts is likely to be higher given the higher energy injury mechanisms discussed

previously[6]. These continued complications highlight the great need for and interest in improved therapies specifically applicable to a recovering combatant.

1.1.4 Heterotopic ossification

In up to 60% of military limb salvage or amputation cases, excess bone formation through heterotopic ossification was noted, typically in muscle or other soft tissue[15]. If the ossification became painful or began to affect joint function, the bony pieces were removed with surgical excision/revision surgery. Military lower-extremity excision rates were initially reported to be 18.7%[16]; in one study that involved closer post-operative monitoring and aggressive early excision, the excision rate climbed to 40%[7]. From the same group, upper-extremity incision rates agreed with the earlier number, remaining at 19%, despite radiographic evidence of HO in up to 60% of the patients. By contrast, a second surgical excision was required in ~6% of HO cases in the civilian population. This suggest an injury mechanism, unique to blast-trauma, which supports the development of HO.

While much is still unknown about the mechanisms that lead to heterotopic ossification, attempts to isolate the cellular causes of combat-associated HO have related the disease to fibrodysplasia ossificans progressiva (FOP), which allows cluster of differentiation (CD)34^+ endothelial cell progenitors (EPCs) to transition to multipotent and eventually ossifying cells through the actions of activin[17]. Similarities in cellular activity and gene expression between FOP and tissue-resident stem cells will be discussed in the section MPCs.

1.2 NORMAL WOUND HEALING

Discussions of dysregulated or enhanced healing rely on a thorough understanding of normal wound healing. Typically used to describe skin repair, wound healing processes apply to many other tissues as the initiating events, infiltrating cells, and cascade of immune- and tissue-modulating factors are common to all injuries. Following that discussion, specific considerations for angiogenesis, osteogenesis, and neurotrophic support will be discussed.

Because of the intimate link between delicate capillaries and all vascularized tissues, normal wound healing begins with an injury-associated blood leakage; in the adult, wound healing ends with the formation of non-functioning fibrous scar tissue[18]. Regardless of the injured tissue, this scar represents a sub-optimal tissue patch, exhibiting different mechanical and functional properties when compared to the surrounding tissue[19].

1.2.1 Initiation: hemostasis and coagulation

Within the first few hours following injury, blood and lymph flush the wound of invading microorganisms and antigens; short times for vasoconstriction couple with extrinsic (tissue factor-factor VIIa-initiated) and intrinsic (platelet-collagen initiated) mechanisms to polymerize fibrin from fibrinogen and thrombin precursors, trapping platelets and their stores of multifunctional factors. Invading leukocytes and platelets release factors triggering: inflammation (interleukin [IL]-1α, IL-1β, IL-6, and tissue necrosis factor [TNF]-α), collagen-synthesis (basic fibroblast growth factor [FGF-2], insulin-like growth factor [IGF}-1, transforming growth factor [TGF]-β), fibroblast-myofibroblast transition (TGF-β), angiogenesis (FGF-2, vascular endothelial cell growth factor [VEGF], hypoxia inducible factor [HIF]-1α,

TGF-β), and support re-epithelialization (epidermal growth factor [EGF], FGF-2, IGF-1, TGF-α)[20].

1.2.2 Migration: inflammation

During the early part of the inflammatory phase, neutrophils and macrophages are recruited and present for 2-5 days if no infection is present; during that time, the neutrophils degrade bacteria and clear necrotic tissue through protease secretion and phagocytosis[20]. Neutrophils release several classes of antimicrobial compounds, including reactive oxygen species, which may also limit the survival and function of resident multipotent cells[21]. In addition, neutrophils secrete VEGF and IL-8, which serve multiple functions including recruiting angiogenic cells and causing neutrophil apoptosis, respectively[20]. Neutrophils can also recruit monocytes and macrophages by releasing some of the same factors secreted by platelets and leukocytes (TNF-α, IL-1β, and IL-6)[18]. Macrophages phagocytose pathogens and cell debris, including apoptotic neutrophils, and can initiate the proliferative phase of wound healing by secreting TGF-β1[22].

1.2.3 Proliferation

Macrophages have the unique power to either promote or resolve inflammation through the production of TGF-α, TGF-β, FGF-2, platelet-derived growth factor (PDGF), and VEGF, which promote cell proliferation and the synthesis of extracellular matrix (ECM)[20]. Through these cytokines, macrophages recruit circulating monocytes and stimulate fibroblasts to proliferate, increasing wound cellularity[23].

1.2.4 Remodeling

Finally, re-epithelialization occurs between 3-10 days after wounding, initiating capillary sprouting and angiogenesis, the growth of new vessels from existing vessels, within the wound. Regulatory cytokines interferon (IFN)-γ and TGF-β promote the deposition of the new ECM, including collagen and fibronectin. Epithelial and non-epithelial cells at the wound edges release EGF, keratinocyte growth factor [KGF], IGF-1, and nerve growth factor [NGF][18].

1.3 VASCULAR CONSIDERATIONS

Vascular function is critical to life on the macroscopic and microscopic scale. Hypoxia or inflammation in the surrounding tissue typically results in the production of VEGF or stromal-derived factor (SDF)-1, which are chemotactic for microvascular endothelial cells (ECs) as they branch from the capillary network[24]. Key events associated with vascular remodeling are summarized in Figure 1[25] and below.

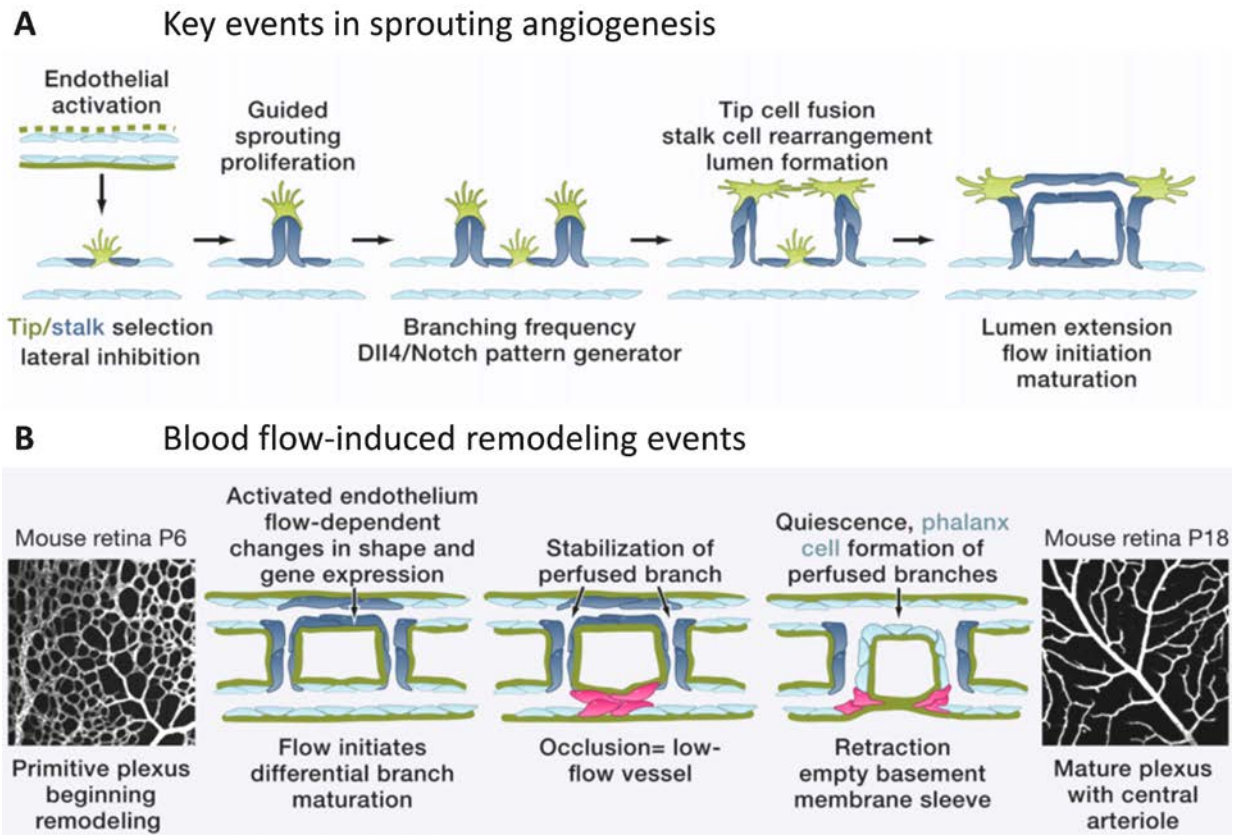

Figure 1: ECs respond to soluble growth factors and blood flow, initiating sprouting angiogenesis

(A) Tip cells sprout and migrate away from existing vessels, guided by factors including VEGF and SDF-1 released by tissues that require vascularization; tip cell fusion and stalk cell rearrangement, followed by (B) blood flow-induced vessel remodeling, results in organized and patent vascular structures. Reprinted with permission from *Cell, 146/6, Potente M, Gerhardt H, Carmeliet P, Basic and therapeutic aspects of angiogenesis, 873-87, 2011, with permission from Elsevier.*

1.3.1 Initiation

VEGF signaling is one of the primary initiators of angiogenic capillary sprouting[26]. Tip cells, expressing VEGF-receptor (VEGFR-2) and low levels of Notch (a transmembrane receptor) but high levels of the Notch ligand Delta-like ligand-4, laterally inhibit neighboring cells from responding to VEGF. VEGF-VEGFR-2 binding, in addition to VEGFR-3 and neuropilin (NRP)-1, initiates filopodia extension by tip cells[27]. These factors signal ECs, normally ensconced within layers of basement membrane and stabilizing mural cells, to degrade the local ECM and become motile. This matrix metalloproteinase (MMP)-mediated degradation, in addition to physically freeing ECs, releases matrix-bound angiogenic factors; some have hypothesized that the released mural cells, or pericytes, form resident multipotent/stem cells that are observed in many tissues[28].

1.3.2 Migration

One tissue-released factor, SDF-1, further stimulates EC tip cells to produce exploratory filopodia and move along angiogenic factor gradients[25]. Attracted by VEGF and SDF-1, tip cells move through the degraded matrix while remaining anchored to trailing, lumen-less stalk cells throughout their explorations; stalk cells, in turn, remain anchored to the fully functioning vasculature[29].

1.3.3 Proliferation and remodeling

Stalk cells have the ability to proliferate and form new branches and tubes, as required by the needs of the tissue. The anastomosis formed by the fusion of two tip cells completes the vascular circuit. The resulting blood vessel network initially forms a disorganized plexus, but subsequent blood flow induces remodeling of the vascular network and maturation of the stalk and tip cells into normally functioning vessel ECs[30]. Mural cells, which may include tissue-resident multipotent cells, attracted by EC-secreted BB isoform of PDGF, wrap around and stabilize the EC networks[24].

1.3.4 Inflammation and angiogenesis regulation

Many factors, especially those released during inflammation including IL-1β and TNF-α, have the ability to disrupt connections in capillary networks. This allows invading blood and immune cells easy passage into the damaged tissue. If prolonged, vascular disruption can restrict normal healing, as is seen with chronic inflammation, keeping tissue in an endless loop of collagenous matrix deposition and fibroblast proliferation with poor vessel organization[31].

VEGF responsiveness is key to angiogenic cell maintenance and function, as well as VEGF's function in other aspects of both wound healing and normal tissue function[32]. This responsiveness can be regulated at several points by the VEGF-responsive cells through the expression of different VEGF receptors[33]. As was discussed above, VEGF binding with VEGFR2 binding typically causes cell migration and proliferation. Neuropilin binding to VEGF may be responsible for the observed migratory effect[29,34]. VEGFR-1, produced in both a membrane-bound signaling and a decoy soluble form, is thought to oppose these effects by

tightly binding VEGF. Membrane-bound VEGFR-1 signals only weakly despite its high affinity for VEGF, sequestering excessive amounts of the molecule[35]. Changes in the relative amount of surface-expression of all of these receptors can change the responsiveness of the target cells to VEGF therapies, as has been noted in cancer models[36].

1.3.5 Experimental models of angiogenesis

Because of the plethora of functions required of ECs, in particular, and the vascular system as a whole, *in vitro* angiogenesis assays test a variety of responses. Many of the assays involve the seeding of ECs onto or inside an extracellular matrix-rich environment[37], typically collagen type I, laminin, or commercially-available growth factor-reduced Matrigel, a tumor-derived ECM. Many cell types will form cords or tubes in this context, depending on the cell seeding density, but EC network formation is particularly dynamic, regressing within a few hours to clustered islands of cells in the absence or blockade of pro-angiogenic stimuli [38].

EC non-specific migration can be examined with the scratch assay[39]. Chemotactic EC-migration can be examined using the Transwell/Boyden chamber[40] assay which restricts cell movement along a factor gradient using a barrier with precisely-sized pores[41]; when ECM is used to coat these pores, further restricting EC migration, zymogen assays or zymograms can help to elucidate specific MMPs responsible for matrix degradation[42].

Occupying a unique position between *in vivo* and *in vitro* assays, the chick chorioallantoic membrane (CAM) assay is another highly sensitive angiogenesis model that allows observation of capillary migration into constructs. Following careful transfer of a chick embryo from the egg shell to a petri dish at incubation day 3, the day 8 shell-less CAM (Figure 2)[43] sandwiches an extensive, dynamic capillary plexus between the inner allantoic and relatively

flat outer chorionic membranes that can be subjected, for up to 4 additional days, to pro- or anti-angiogenic stimuli[44]. Multiple factor- or cell- depots, as well as appropriate controls, can then be exposed to identical oxygen tension and systemic embryonic circulation[45]. It is possible to test the angiogenic activities of cells sourced from widely-varying species, including humans, with the CAM assay since the chick immune system is not yet fully developed[46]. To minimize cell migration and maximize the observable CAM vessel recruitment, depots should restrict the movement of encapsulated cells. This allows the free diffusion of secreted factors and invasion of the relatively small avian vessels.

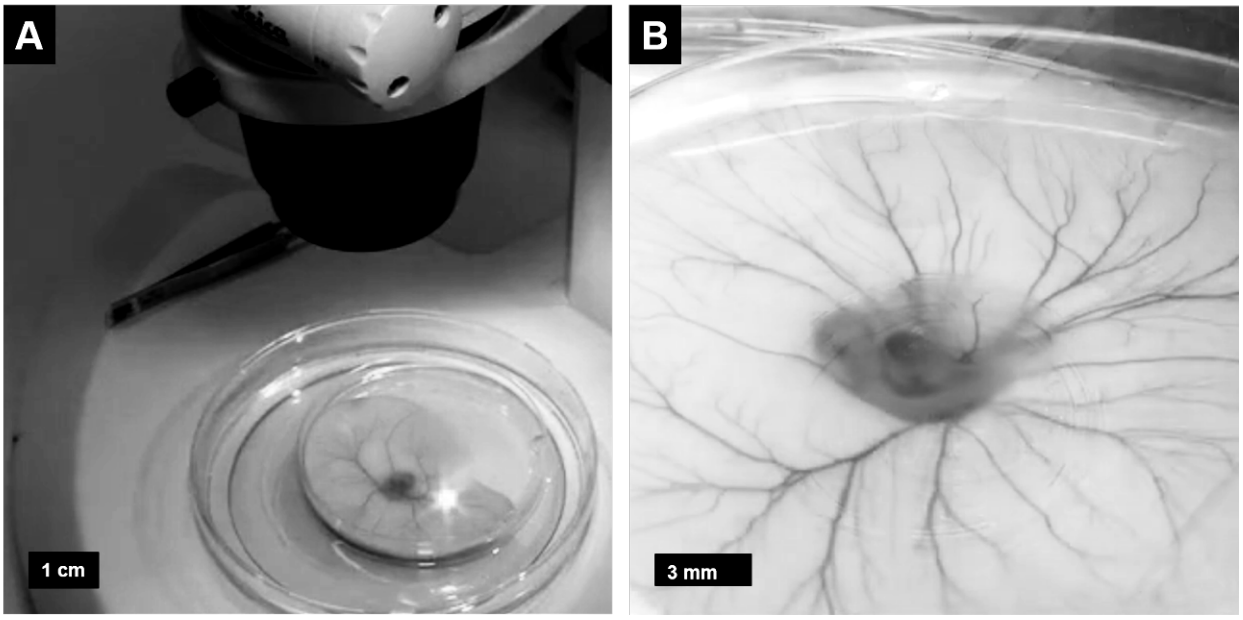

Figure 2: Chick embryonic chorioallantoic membrane (CAM) assay system.
The (A) shell-less/ex ovo CAM assay offers a wide viewing surface on which several growth factor- or cell- depots may be deposited. Vasculature supplying the embryo (B) is easily visible under microscopy. Embryos can remain viable for up to 16 days ex ovo; with permission from JoVE, modified from Yalcin, et al [43,47].

1.4 PERIPHERAL NERVE CONSIDERATIONS

Nerves and blood vessels are intimately connected, both in development and in the adult[32]. They exhibit structural similarities and signal using some of the same molecules. Macroscopically, nerves bundle multiple individual neurons, with supporting Schwann cells, protective sheaths, and blood vessels, into neurovascular units[48]. Nerves and blood vessels regulate their responsiveness to paracrine signals, especially VEGF, by adjusting production of both decoy receptors, such as VEGFR-1, or soluble forms of active receptors such as sVEGFR-2[49] or sNRP1[50]. This suggests that pro-angiogenic therapies involving VEGF might also positively affect nerve growth and extension. Single neurons are encapsulated by Schwann cells which secrete trophic and maintenance factors in addition to protective myelin. Successive bundling of nerves and blood vessels into the endoneurium, perineurium, and epineurium maintain physical and nutrient support of the nerves[51].

1.4.1 Peripheral nerve injuries with combat considerations

Nerve injuries associated with combat-related trauma contribute to compromised functional outcomes and major disability[52,53]. In terms of the final outcome, one study found that in a cohort of patients that sustained upper limb nerve injuries, persistent weakness (84%), sensory deficit (69%), and chronic pain (24%) resulted from the injury[54]. In an earlier study of patients requiring vascular repair following a gunshot injury, 39% achieved a normal extremity, compared with only 7% if there was an associated peripheral nerve injury in the same limb[55]. As with poor limb function outcomes, peripheral nerve injury-induced morbidity resulted in reduced military preparedness, early discharge, poor reintegration into civilian life, and may contribute to

the wounded warriors' higher rates of depression and suicide. A summary of the relevant aspects of nerve injury and healing is described below.

1.4.2 Classification of nerve injuries

Nerve injuries are classified in terms of degree of injury using the Seddon (3-point) or Sunderland (5-point) scale[56,57]. The lowest scores indicate damage to axons without axonal severance; if the injury is allowed to heal, return of nerve function is likely. Scores ≥3 on either scale indicate nerve damage that will be slow or unlikely to heal as the damage has extended from individual neurons to the nerve structure. Healing is summarized in Figure 3[58] and below.

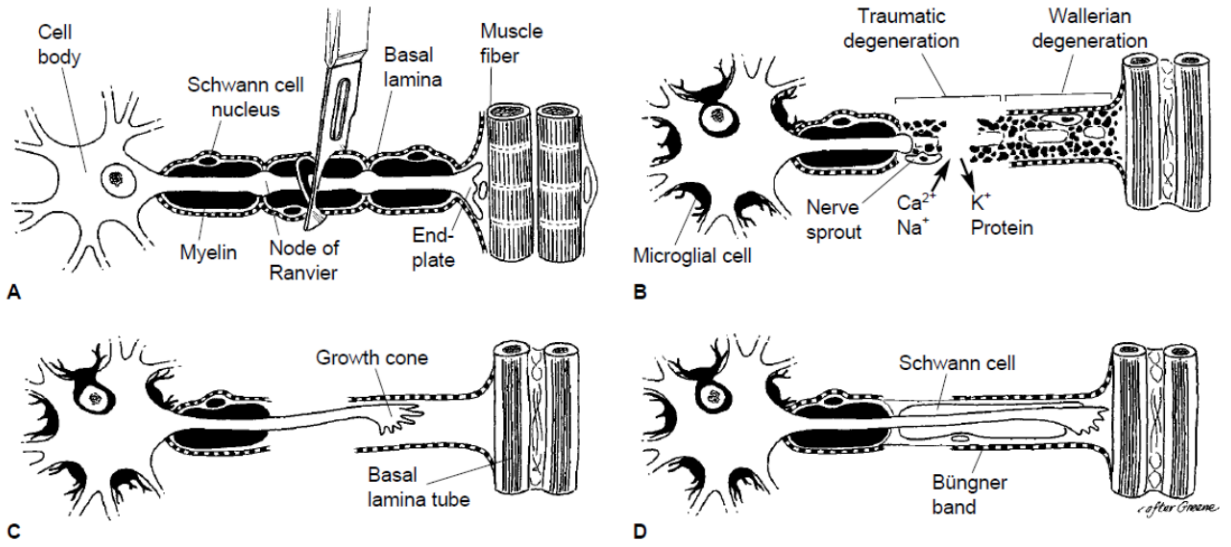

Figure 3: The four stages of nerve healing following injury rely on the activities of Schwann cells
Following injury (A), Schwann cell-mediated degradation (B) clears the way for the extending axon(C). Schwann cells secrete a provisional matrix, termed bands of Bungner, which guides the growth cones of extending axons (D). Throughout this process Schwann cells secrete several factors to recruit nerves or sustain nerve survival. Reprinted with permission from *Muscle and nerve, 13/9, Seckel B, Enhancement of peripheral nerve regeneration, 785-800, 1990, with permission from Elsevier.*

1.4.3 Migration/Stage I

Although less pronounced than in other systems, nerve tissue migration initially involves retraction and sealing of the proximal and distal ends of the broken nerve axons. Additionally, Schwann cell-mediated, calcium-dependent degradation of the cell material, particularly myelin, contribute to Wallerian degeneration[51]. Currently, nerve regeneration is limited to the migratory capacity of the proximal nerve stump; that stump is most effectively recruited by factors released by the distal stump, discussed below[59]. Assuming neuron cell body survival, proximal stump migration continues throughout the nerve healing process and can last up to two years[60].

1.4.4 Inflammation/Stage II

The remaining Schwann cells release cytokines that promote inflammatory cell infiltration and damaged tissue clearance, discussed above. Myelin clearance is especially critical to the eventual growth of nerves[61]. Once cleared, the lesion defines the gap that must be crossed by sprouting, exploratory axons to re-innervate the target organ; the maximum growth rate of neurons has been observed to be about one inch per month[62]. This slow growth rate may be further limited by fibrotic scar formation, and over extended time, correlated to the length of a critically-sized nerve defect (3-5 cm)[63], the target tissue and Schwann cells may lose their abilities to support reinnervation[64].

1.4.5 Proliferation/Stage III

Schwann cells next proliferate, emerging from the proximal and distal axon stump and repopulating the gap leading to the distal nerve end. After a sufficient number of Schwann cells repopulate this area, they begin to align across the gap, forming bands of Büngner that serve as a scaffold to guide sprouting axons to the end organ. Scar formation can block this process and lead to painful neuromas and distal tissue atrophy.

1.4.6 Remodeling/Stage IV

Schwann cells within the bands of Büngner secrete a number of neurotrophic factors that assist with axonal sprouting, guidance and survival. These factors include NGF, brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and glial cell line-derived neurotrophic factor (GDNF)[65]. Importantly, Schwann cells also produce VEGF which supports both the vasculature supplying the nerve and the neurite extensions themselves, which express both VEGFR-2 and NRP-1[66]. These cytokines have been shown to be important for sciatic nerve regeneration and remyelination, as well as survival of other motor and sensory neurons[67].

1.4.7 Standard treatment of nerve injuries

Three autologous strategies are currently used to treat nerve injuries: (1) direct nerve repair (i.e., proximal-to-distal end tensionless suturing), (2) the gold standard neural autograft repair, in which a nerve of similar type and size is excised and interposed between the proximal and distal

ends of the severed nerve, and (3) biological autograft repair, in which another tissue such as an artery or vein is placed within the gap to support nerve repair.

Although ideal, direct nerve repair (Strategy 1) is rarely possible in both civilian and military nerve repair applications as it is only indicated in cases of sharp nerve division with minimal gap (<2.5 mm), intact blood supply, and soft-tissue coverage. Autologous grafting of neural or vascular tissue (Strategies 2 and 3) may result in donor-site morbidity and are associated with recovery rates of just 50%[68]. For the blast-injured combatant, adequate donor tissue may not be available.

1.4.8 Alternative strategies for nerve repair

While size-matched allogeneic nerves or blood vessels[69] may be utilized in civilian populations as a form of nerve graft, graft acceptance requires long-term immunosuppression that could prove fatal to the blast-injured patient facing severe infection risks. Sterile nerve guide conduits are an alternative surgical treatment option that does not further tax the patient but are associated with poorer outcomes; several strategies have been employed clinically to maximize patient functional recovery[70], as detailed below.

Improvements aimed at treating peripheral nerve damage usually involve increasing the degree to which the synthetic scaffold mimics native, healthy nerve architecture, cell content, or Schwann cell growth factor content. Clinically-approved nerve guide conduits vary widely in terms of their composition, but their most basic function isolates the damaged nerve, concentrating neurotrophic signals from the distal stump while eliminating unwanted, scar-forming immune cell infiltration[71]. This cellular isolation must allow constant nutrient exchange for the sensitive nerves; these competing concerns have been addressed in recent years by tightly

controlling the porosity and thickness of nerve guide conduits[71]. The wall of these conduits, either pre-fabricated tubes or wraps, must be strong enough to retain suture and avoid collapse yet soft, flexible, and biocompatible (eliciting little or no immune response) to prevent inflammation[63]. While some nerve guide conduits are made of material that is not intended to degrade, such as poly-vinyl alcohol (PVA), silicon, or poly-urethane (PU), work in recent years has led to the creation of several commercially-available biodegradable nerve guide conduit scaffolds composed of poly-glycoli c acid (PGA), poly-lactic acid (PLA), poly-caprolactone (PCL), or some combination of the FDA-approved polymers. These polymer conduit scaffolds degrade through hydrolytic bond cleavage at successively slower rates thought to allow gradual replacement by native tissue[63].

Because of their solubility in organic solvents, these polymers are also easily manipulated into desired shapes, such as aligned fibers created through electrospinning, that provide physical guidance and potentially increase the efficiency of extending neurites[72]. Biologically-derived materials like fibrin may also provide this physical link; fibrin composes the natural bridge linking two nerve stumps during normal nerve repair, collagen or gelatin (denatured collagen), chitosan, keratin, hyaluronic acid, and even silk fibroin have been incorporated into nerve guide scaffolds with intriguing results in animal models thought to be due to nerve cell-ECM interactions[72]. Such soft molecules fail to protect nerves from mechanical damage and degrade on the order of days, weeks, or, at best, months when therapeutic intervention may be required for several months or years[51,63,73,74]. Hybrid scaffolds may utilize various combinations of synthetic and natural biomaterials to precisely tune the degradation rate and ECM signaling molecules[72].

Stem cell or growth factor augmentation of these guides remains a popular topic of research. This includes the incorporation of bioactive neurotrophic factors - including NGF, BDNF, neurotrophin-3 (NT-3), GDNF, and IGF-1, or Schwann cells/Schwann cell-mimics that express these factors[69,75–78]. Stem cell transplantation may affect these functions through an immunomodulatory, paracrine effect.

1.5 BONE CONSIDERATIONS

Bone fracture healing is another process that is delicately balanced with active angiogenesis. Among the tissues discussed, bone is unique in its potential to heal with virtually no scarring when injured in the adult[30]; however, vascular disruption is associated with delayed or non-union of bony fractures. In addition, inflammation plays a role in bone formation or destruction[79]. Extensive *in vitro* and *in vivo* work has investigated bone formation over the years, leading to an understanding of the necessary gene expression and matrix-production timecourse required for proper bone formation and healing.

1.5.1 In *vivo* osteogenesis during fracture repair

In the adult, bone forms chiefly through endochondral ossification. During endochondral ossification, a cartilaginous matrix is deposited, and subsequent remodeling, chondrocyte-changes including hypertrophy, and, eventually, mineralization produces the mineralized collagen structure known as bone. The process is closely associated with vascular remodeling[80].

In vivo, normal bone repair relies on a dynamic balance between matrix deposition and matrix resorption, carried out by the balanced activities of osteoblasts and osteoclasts, respectively, among other cell types. Dysregulation of either cell types leads to pathology. Osteoclasts, derived from hematopoietic $CD34^+$ precursors, are recruited to the site of injury/fracture, and their release of embedded factors following mineral- and collagen-matrix degradation signals the beginning of a cycle of bone formation[81]. They mature in response to receptor activator of nuclear kappa-B ligand, among other molecules. Osteoblasts, committed matrix- and mineral-producing mesenchymal cells, express genes roughly following the timecourse detailed in [82], generated to describe *in vitro* osteogenesis, with a few exceptions. The addition of TGF-β family members bone morphogenetic proteins (BMPs) -2, -4, and -7 to factors released during wound healing promoted bone formation and osteoblast maturation[83]. Although it can stimulate mature osteoblasts to maintain their osteogenic phenotype, activin opposes bone formation by encouraging the function and maturation of osteoclasts; its actions can be opposed by inhibin or follistatin, which are released primarily by the gonads[84,85]. In addition, Indian hedgehog and parathyroid hormone-related peptide as well as their induced factors play strong roles in osteogenesis[86].

Within the osteoblasts, runt-related transcription factor-2 (RUNX2) increases, eventually driving the expression of osterix, osteocalcin (OCN), and bone sialoprotein-II (BSP), among other molecules, which are deposited within the newly-formed collagen-I (COL-I) matrix[87]. Prior to this mature, matrix and mineralizing phenotype, BMP2 signaling is necessary for observed increases in production of these matrix-deposited factors, especially in muscle-derived osteoblast progenitors[87], but excessive amounts can cause pathologies[88]. Hydroxyapatite minerals composed of calcium phosphate are nucleated by both collagen and collagen-associated proteins

as the immature, woven bone forms, with the necessary phosphates supplied by osteoblast-supplied alkaline phosphatase (ALP)-phosphate conversion. These changes are summarized in Figure 4[82]. Recent work suggests that ALP gene expression, typically used as a marker of early osteogenesis, peaks twice in vivo; it is therefore more appropriately called a later osteogenesis markers; additionally, COL-I expression by osteoblasts decreases over time[89].

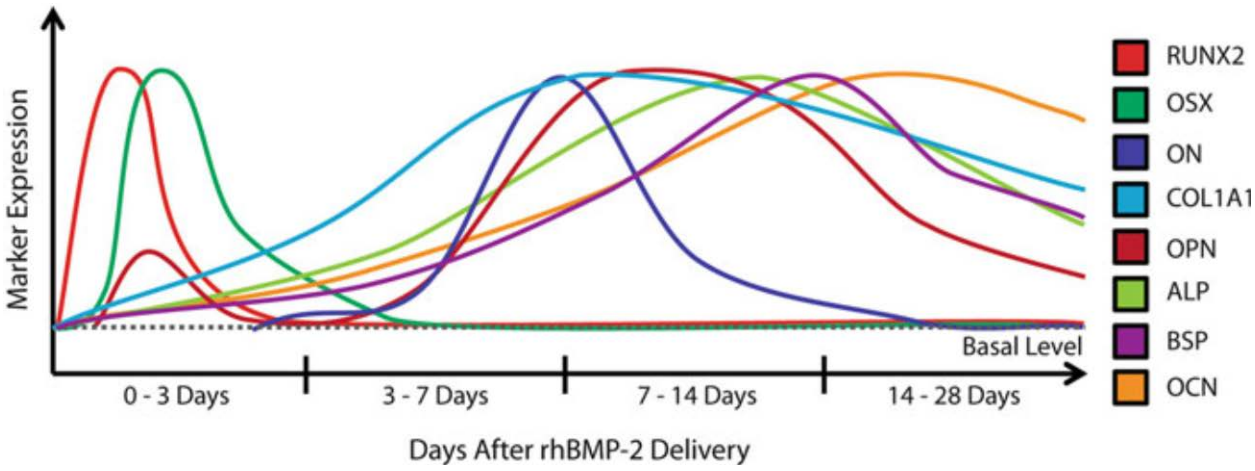

Figure 4: Canonical osteogenic gene expression *in vitro* and *in vivo; reprinted with permission*[82]
Exogenous BMP-2 accelerates these changes but is not necessary given dexamethasone, ascorbic acid, phosphate-supplemented culture. *In vivo*, recent work examining gene expression more closely indicates that RUNX2 gene expression typically follows the pattern observed for OPN; additionally, COL-IA1 gene expression decreases nearly constantly throughout the culture period. The observed differences are likely due to the selection of primers (and if applicable, probes) for gene expression analysis[89].

1.5.2 *In vitro* considerations

Canonical *in vitro* osteogenesis mimics osteoblast function and begins with dexamethasone-driven decreases in fibroblast/multipotent cell production of IL-6[90]; the loss of this stem-ness factor allows the COL-I-producing cells to release increasing amounts of calcium[91]. This excess calcium permits the formation of insoluble hydroxyapatite crystals upon conversion of exogenous phosphates by ALP. The crystal deposits serve as nucleation points for more crystal deposition, eventually forming visible mineral deposits[92]; RUNX2 again drives the markers of mature osteogenesis[93].

Apparent *in vitro* osteogenesis is sensitive to a variety of factors. Alkaline phosphatase may be expressed and present but inactive; necrotic cells or normal cell debris or excess culture phosphate can also nucleate mineralization[94,95]. While outside the scope of this work, three-dimensional, particularly pre-mineralized matrices have received a great deal of attention in recent years, especially because of their potential utility in healing of bone non-unions; robust osteogenic responses were observed when both the bone and the vasculature were considered in the construct formation[80] although specific cell contributions become difficult to distinguish.

1.5.3 Osteogenesis and angiogenesis

Endothelial cell and osteoblast interactions are critical for bone formation *in vivo*. Connexin 43 enables direct cell-to-cell contact between the two cell types, and subsequent work indicated that the rate of bone marrow- mesenchymal stem cell mineralization may be directly controlled by endothelial cells[96]. Similarly, osteoclast recruitment, as for neutrophils and macrophages during

wound healing, is controlled by the migration of osteoclasts across the thin, endothelial-blood vessel membrane[80].

1.5.4 Osteogenesis and inflammation

Lin, et al., found a dose dependent biphasic response of rat neonatal calvarial cells to IL-1β treatment. Short treatments (<48 hours) with IL-1β increased bone nodule formation, but longer treatments (>4 days) suppressed it. Doses between 1 pg/ml and 10 ng/ml increased early nodule formation and mineralization but ultimately adversely affected total nodule formation[97]. In human MSC cultures, Ferreira, et al., found that MSCs form hydroxyapatite, excessively in the presence of IL-1β through the activity of ALP[98]. Any clinical intervention or implant is likely to begin the wound healing process anew, stimulating significant local inflammation, so an understanding of the limitations of MSCs within the context of inflammation could contribute to more useful, directed wound healing.

1.6 ADULT STEM CELLS IN TISSUE REPAIR

Stem cells are defined as a self-renewing population of cells whose progeny can be differentiated to perform specific functions. Totipotent stem cells, such as the fertilized human egg, can generate every tissue of the body; pluripotent and multipotent stem cells are progressively restricted in their ability to differentiate into multiple tissue types[99]. The best characterized multipotent cells are mesenchymal stem cells. Their plasticity with respect to differentiation and recent clinically-relevant advances in identifying, isolating, and concentrating mesenchymal

stem cells[100] is extremely exciting in the context of tissue repair. A brief history of the use of mesenchymal stem cells with respect to tissue repair is discussed below.

1.6.1 Ultimate *in vivo* stem cell sources

Any discussion of adult, tissue-specific stem cells must acknowledge several competing viewpoints. One viewpoint holds that multipotent progenitor cells are ultimately derived from the bone marrow. Such cells are mobilized into the blood stream upon systemic release of inflammatory or stress factors and home to the site of injury[101] following chemotactic gradients (stromal derived factor-1 is implicated) [102]. Another viewpoint holds that adult stem cells are a ubiquitous pericyte-like cell type that can be released by local inflammatory or stress factors; a third viewpoint suggests a non-vessel-associated resident stem cell[103]. Regardless of the ultimate origin of these adult multipotent progenitor cells, these multipotent stem cells can act to regulate inflammation and, once present in the damaged tissue, are thought to participate in tissue healing. Stem cell sourcing has become a topic of great interest in recent years, and military-specific interest has extended to stem cells derived from allogeneic ocular, neural, cardiac, skin, muscle, and mesenchymal sources among others[6].

1.6.2 Defining mesenchymal stem cells

Following their original discovery and several re-definitions in the 1990s, mesenchymal stem cells, without source qualifications, were officially defined in 2006[104]. Experimentally, mesenchymal stem cells are identified first by their ability to adhere to plastic. Next, proliferative and differentiable mesenchymal stem cells are identified, at a minimum, by the expression of a

combination of surface markers including STRO-1, SB-10, SH3 (cluster of differentiation [CD]73) and SH4 antigens as well as Thy-1 (CD90), TGF-β receptor type III endoglin (CD105), hyaluronic acid receptor CD44, integrin a1 subunit CD29, activated leukocyte-cell adhesion molecules (CD166), and possibly others[104]. MSCs are negative for the hematopoietic markers, CD19/CD79a, CD34, CD45, CD11b/CD14 and human leukocyte antigen (HLA)-DR[105]. CD73, SH4 and STRO-1 antibodies recognize antigens that are present on MSCs and other cells but not hematopoietic cells[106]. The presence of CD75, CD90, and CD105 and absence of hematopoietic markers, while not sufficient to fully identify a mesenchymal stem cell, has been suggested by the International Society of Cell Therapy as the minimum positive criteria necessary to consider a potential cell a mesenchymal stem cell[104]. Density gradient separation of mononuclear cells from blood or minced or digested tissue can help to purify the above population, but it remains a challenge to isolate pure and unequivocal mesenchymal stem cells from a mixed cell population[107].

1.6.3 Autologous versus allogeneic tissue sourcing

The immune system bestows on the human body the ability to prevent or suppress pathogen infiltration; broadly, inflammation recruits the first line of defensive, innate immune cells while continued antigen presence primes the antibody-associated adaptive immune response[108]. While these protective functions are useful to combat pathogens, immune rejection can compromise cell- or biomaterial-based therapeutic strategies. Exciting evidence suggests that stem cells derived from multiple sources may be privileged when it comes to both types of defense[109]. Mixed lymphocyte reaction studies suggest that mesenchymal stem cells can suppress the proliferation of those cells in the presence of a known antigen. Additionally, mesenchymal stem

cells normally express only one of two Major Histocompatibility Complex (MHC) molecules used by the immune system to recognize foreign cells, as they lack HLA-DR expression unless activated by stress[110]. The combination of these and other findings has led to the use of mesenchymal stem cells in clinical trials that attempt to combat graft-versus-host disease, with varying success,[109] and suggests that allogeneic, disease- and activity-screened, stem cells might be utilized therapeutically.

Contrasting this statement, mesenchymal stem cells can express both MHC molecules if activated by stress, a likely scenario given the exigencies of the healing tissue environment. More recent work suggests that mesenchymal stem cells are therefore immune-evasive, rather than immune-privileged[111], injecting a note of caution into the use of allogeneic mesenchymal stem cells. Even without possible immune rejection, storage and supply of tested mesenchymal stem cells is likely to be costly and difficult for combat-associated hospitals. This suggests that autologous mesenchymal stem cells would be safer for the patient and easier to implement clinically.

1.6.4 Bone marrow-derived mesenchymal stem cells (MSCs)

First described by Friedenstein in 1965, bone marrow-derived mesenchymal stem cells (MSCs)[112] were initially identified for their ability to form bone when transplanted heterotopically. Through work by Owen[113] and, subsequently, many others, the common origin of adipogenic, osteogenic, and chondrogenic cells from bone marrow precursors was established[114]. Subsequent work has found some form of differentiable stem-like cell in nearly every tissue of the body[115]. MSCs are thought to home to sites of injury by following SDF-1 gradients; once present at the site of injury, MSCs work via paracrine mechanisms to draw in

immune cells using IL-1β while dampening the severity of other inflammatory factors through IL-10[116]. They additionally secrete hepatocyte growth factor (HGF), epidermal growth factor (EGF), and VEGF that can act to promote the proliferation of resident cells and, depending on the context, more or less vascular organization[117]. A very small number of MSCs will engraft or fuse with native cells at the injury site.

In terms of limb reconstruction, bone marrow are perhaps the ideal candidates to use for structural repairs as they were first identified as bone-forming cells[112]. Their angiogenic properties are thought to be positive due to high secretion of VEGF and SDF-1[107], although a large body of work also examines their anti-angiogenic effects on diseases such as cancer, possibly finding action through thrombospondin-1 and VEGFR modulation[118]. MSCs are particularly difficult to isolate, requiring a painful iliac crest isolation from a healthy donor or the recovering patient.

1.6.5 Adipose-derived mesenchymal stem cells (AD-MSCs)

Although MSCs are described as the clinical and research gold standards, particularly in the area of bone formation; effective multipotent cells can be isolated from a growing list of tissue once deemed to be surgical waste, including adipose tissue, deciduous teeth (dental pulp), and birth-associated tissues (umbilical cord and placenta), among others[119,120]. The isolation methods for these tissue are typically less invasive than for MSCs, supporting patient recovery, and depending on the application, such surgical waste-derived multipotent cells may be more effective, therapeutically, than MSCs[121].

Among these alternatively-sourced stem cells, adipose-derived mesenchymal stem cells (AD-MSCs) are the most popular and best characterized, as evidenced by their successful

transition from research to clinical use[120]. AD-MSCs and other microvascular cells are known to support nerve growth and regeneration[122] as well as EC angiogenic function. Osteogenic differentiation of AD-MSCs is less robust than MSCs[123], but it is still strong enough to warrant clinical trials to aid bone fracture repair[124].

1.6.6 Muscle-derived stem cells

With regards to source-related properties of previous tissues, a great deal of work has been published using human-sourced material that has shown promise for clinical translation. Growing evidence suggests that skeletal muscle contains or recruits all cell types necessary for limb function[125]. Satellite cells, a $CD34^+$ muscle-derived multipotent cell distinct from pericytes or mesenchymal stem cells, have been noted since the 1950s and have been shown to possess adipogenic and osteogenic differentiation abilities[126]. Additionally, Qu, et al[127], identified a population of slowly-adhering muscle-derived cells possessing multipotent activities and better long-term survival. In subsequent work, osteogenic and, more recently, nerve support were explored in mouse models[125,128]. A $CD34^+$ subset, labeled myo-endothelial or vascular endothelial progenitor cells, exhibited marked angiogenic activity[129].

In extensive work in mice, Birbrair identified a population of $Tuj1^+$ (a neuron-specific beta tubulin) cells derived from $Nestin^+$ muscle pericytes[130]. $Nestin^+$ pericytes induced *in vitro* angiogenesis and participated in tumor perfusion[131]. Again highlighting the close interactions of the nervous and vascular systems, this finding also suggests that muscle-derived multipotent cells, particularly the mixed populations likely to be present in primary isolates, would support both nerve and vascular functions *in vivo*.

Survival and tissue repair takes up the majority of the physical resources of a recovering blast-traumatized patient. Additional surgeries to isolate the above stem cell populations would likely place an undue, possibly unsustainable, burden on the already-taxed patient.

1.6.7 Traumatized muscle-derived multipotent progenitor cells

Within the subset of muscle-derived stem cells, MPCs deserve particular note, given their essential role in this dissertation work. It was first noted in 1986 that traumatized muscles secrete factors that influence the proliferation of reparative satellite cells[132]; current work and exercise science assert that small injuries and incremental repairs are necessary for ultimate restoration of tissue function[133]. First reported in 2008, MPCs were derived from the second- or third-debridement of wounds of blast-traumatized soldiers who were treated within 3-7 days of their initial blast injury; fat- and fascia-free healthy muscle tissue was obtained from the thin margin removed during debridement. In the presence of copious amounts of antibiotics, plastic-adherent MPCs were filtered from collagenase type II-digested minced muscles. Initial and subsequent work showed that these cells were positive for the stem cell markers and negative for hematopoietic, lymphocyte and leukocyte markers, discussed in 4.2.8; additionally, cells were positive for CD29, CD44, and CD146, indicating cellular and matrix adhesion molecule surface expression as well as endothelial cell markers[5].

In addition to standard adipose and chondrogenic differentiation, these cells were shown to be capable of osteogenic differentiation, suggesting similarity in function and multipotency to MSCs. Because of interest in their contribution to HO, extensive *in vitro* characterization has been carried out. To summarize the work presented in several papers: Total ALP production was similar between MPCs and MSCs, but normalized (per-cell) ALP activity was lower in MPC

cultures by day 14. Osteogenic MPCs expressed RUNX2, ALP, and OCN differentially from growth cultures. MPC culture mineralization began at 21 days and grew stronger after 28 days, but this mineralization was moderate when compared to MSCs[5,134]. EPCs make up an abundant fraction of the cells isolated from muscle immediately following digestion and have been implicated as the key initiators of FOP, a slow-onset, genetic form of HO[17,135]. These EPCs secrete TGF-β1-3 at detectable levels[135,136] which may further enhance or suppress the observed osteogenesis *in vitro* depending on their concentration, duration of activity, and affected cells[89,137,138]. MPCs were shown to express genes for inflammatory markers that affect cell differentiation, including IL-1β and IL-6, but this expression was not statistically significant from non-traumatized controls or MSCs[135,139]. Critically, for this work, MPCs were demonstrated to express and produce VEGF at similar levels to MSCs[139]. Later work found key increases in BMP6 expression by MPCs derived from patients who developed HO over those who did not[136].

For neurotrophic differentiation, MPCs, were shown to transdifferentiate across lineages to a neuroglial-like phenotype; this phenotype proved as adept as MSCs at promoting the growth of peripheral nerves on tissue culture plastic[140]. MPCs expressed and produced BDNF and Nestin, markers of neurotrophic and neural activity, respectively, under a specialized neurotrophic induction protocol[141]. Induced cells also displayed increased CD56 presentation, a molecule important for MPC-nerve cell interactions[141].

1.6.8 Key differences between MSCs and MPCs

MPC differentiation along the adipogenic-osteogenic axis was dependent on activin-A which MPCs produced preferentially to MSCs in addition to lower follistatin (an inhibitor of activin-A)[142], suggesting a weighting towards an osteogenic phenotype. Peroxisome proliferator-

activated receptor (PPAR)-γ, an indicator for adipogenesis, was differentially upregulated in MPC osteogenic culture compared to MSC. Subsequent work examining HO nodules noted the presence of brown- and white-fat markers and the ability of distinct MPC subpopulations, with high endothelial marker expression, to preferentially differentiate along an adipogenic pathway[5,143]. Although no mechanism for the difference was investigated, MPC-suppression of mixed lymphocyte proliferation, an indicator of MPC immunocompatibility, was less potent than that of other types of multipotent stem cells including MSCs[139].

When compared with normal non-traumatized muscle, MPCs express higher ratios of TGF-β3 to TGF-β1, indicating a fibrotic/matrix-producing phenotype[15]. BMP11 and BMP4, associated with bone/muscle and bone/cartilage differentiation, respectively, were upregulated, while BMP3B, NODAL, and myostatin, associated with DNA methylation, embryonic development, and muscle differentiation, were downregulated[89,135,144]. In one study examining osteogenic genes by PCRArray, MPCs expressed higher amounts of RUNX2 but lower ALP and OCN than MSCs when each was normalized to their respective growth controls[134]. Taken together, these differences suggest a cell population primed to form either osteogenic or fibrotic tissue. The ultimate fate of MPCs likely depends on their environment, a dependence that will be explored in the work described below.

1.7 HYPOTHESIS AND SPECIFIC AIMS

Ongoing research suggests that inflammation alters the ability of cells to respond to their environment, sensitizing them to some signals and dampening the influence of others[145]. Because of the inflamed nature of their environment just prior to isolation, MPCs represent a uniquely

stimulated cell source. Although frequently compared to MSCs in terms of activity, extensive work has gone into characterizing the regenerative potential of MPCs and noted key differences between MPCs and MSCs. These differences are likely critical in regulating their biological activities under the abnormal or pathophysiological conditions seen in a traumatic wound, and further investigation of these differences could expand the potential pathological or therapeutic implications for MPCs. To date, no detailed studies have examined MPC activities, either pathologic or therapeutic, in the context of their altered isolation/implantation environment or of paracrine signaling.

The overall hypothesis is that MPCs possess regeneration- and clinically-relevant trophic and -functional activities capable of influencing angiogenesis, neurite extension, and osteogenesis, and that these activities are altered by the physical and cellular microenvironment of the MPCs. The following specific aims were designed to test this hypothesis.

Specific Aim 1: Determine the ability of MPCs to promote angiogenesis by examining MPC-influenced EC-organization *in vitro* and MPC-recruitment of capillaries to an implantable construct *in vivo*.

Specific Aim 2: Examine alterations in MPC neurotrophic activity due to EC-influence and structured biomaterial scaffolds.

Specific Aim 3: Determine the regulatory effect of IL-1β-simulated inflammation and EC-influence on MPC osteogenic differentiation.

The following chapters detail the results of each specific aim.

2.0 TRAUMATIZED MUSCLE-DERIVED MULTIPOTENT PROGENITOR CELLS RECRUIT CAPILLARIES AND STIMULATE ANGIOGENESIS THROUGH VASCULAR ENDOTHELIAL GROWTH FACTOR-A ACTION

2.1 INTRODUCTION

Along with a critical supply of nutrients, capillary networks composed of microvascular endothelial cells (ECs) allow the invasion of various blood-delivered immune and multipotent cells, possibly bone marrow-derived mesenchymal stem cells (MSCs), that can augment the muscular, osteogenic, and neural functions of the healing limb[6,146]. Additionally, capillary networks are thought to be maintained or disrupted through the interactions between ECs and resident, vessel-associated MSCs/pericytes[147].

Normal vascular function of ECs is influenced by a variety of external and internal factors that exquisitely balance opposing signals promoting vessel sprouting and vessel regression[31]. One such molecule is vascular endothelial growth factor (VEGF), which has been effective at helping patients in the clinic to recover from ischemic coronary episodes[148]. VEGF acts synergistically with platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and angiopoietin (ANGPT) in various models to enhance collateral vessel development[149–151]. On a cellular level, VEGF is critical to EC survival and maintenance of normal vessel function[152,153], and in tip cell EC sub-populations, VEGF increases cell motility, proliferation, and migration[154].

When cultured under various conditions *in vitro*, MSCs have been consistently reported to secrete VEGF, among several other factors that can tip the balance towards sprouting angiogenesis. Present in detectable levels within MSC-conditioned medium (CM), these other factors include: angiogenin, ANGPT-1 and -2, chemokine (C-C) ligand (CCL)-2, chemokine (C-X-C) ligand (CXCL)-7, FGF, hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1, interleukin (IL)-6, nitric oxide (NO), PDGF, stromal-derived factor (SDF)-1, and tissue inhibitor of metalloproteinase (TIMP)-1 and -2[155–160]. Among these, MSC-produced VEGF and FGF appear to be the most potent angiogenic molecules present in MSC-CM; their combined activities can account for up to two-thirds of observed MSC-CM support of EC proliferation and migration[161]. VEGF alone may constitute up to half of the total MSC-CM support of EC network organization[162].

Recent work has demonstrated the production of VEGF by MPCs and the positive effect of MPC-CM on EC proliferation[139]. As a first step towards assessing the potential and viability of MPC-based therapies, the study reported here attempted to address the effect of MPCs, compared to MSCs, on angiogenesis. Two angiogenesis assay platforms were utilized to test the effect of MPC secretome, focusing on VEGF, based on the ability of ECs to form branched networks with one another *in vitro* and the chemotactic effect and the recruitment activity of MPCs on capillaries *in vivo*.

Results presented below showed that, in a Matrigel EC cord-forming assay[38], conditioned medium derived from MPCs (MPC-CM) was pro-angiogenic through the activity of VEGF. MPCs also recruited small-caliber blood vessels in the chick embryonic choriollantoic membrane (CAM) *in vivo*, in a manner dependent on cell density and other factors besides VEGF. Taken

together, these findings support the potential therapeutic value of MPCs for the promotion of angiogenesis and also illustrate biological differences between MPCs and MSCs.

2.2 METHODS

2.2.1 MPC isolation

MPCs were harvested from human muscle tissue specimens obtained following debridement of blast-traumatized wounded US military personnel with Institutional Review Board (IRB) approval (Walter Reed National Military Medical Center). Fascia-free minced muscle tissue (200 μg) was enzymatically digested for 2 hours (Dulbecco's Modified Eagle's Medium, DMEM; GIBCO; 0.5 mg/ml collagenase 2, Sigma) at 37°C under gentle agitation, as originally described by Jackson, et al[163]. MPCs were strained through a 40 μm cell strainer and pelleted by centrifugation at 200 x g for 5 minutes. Cells were resuspended in DMEM, 10% fetal bovine serum (FBS; GIBCO), and 5% penicillin-streptomycin (PS; GIBCO), plated in a T150 tissue culture flask (Corning), and allowed to adhere overnight. Cells were then washed extensively with phosphate-buffered saline (PBS; GIBCO) and cultured in DMEM, 10%FBS, and 3% antibiotics-antimycotic (PSF: penicillin-streptomycin-Fungizone; GIBCO), at 37°C under 5% CO_2 until confluent.

2.2.2 MSC isolation

Bone marrow-derived MSCs were obtained after flushing scissor-minced trabecular bone removed from the femoral heads of patients undergoing total hip arthroplasty (IRB approval, University of Washington). The cell-enriched rinsing medium (α-Minimal Eagle's Medium, α-MEM; GIBCO; 1% PSF) was strained through a 40 μm cell strainer, and the resulting cells pelleted at 300 x g for 5 minutes. Cells were washed with rinsing medium and pelleted twice before re-suspension in growth medium (rinsing medium + 10% FBS + 1 ng/ml FGF-2; Fisher). Cells were allowed to adhere to T150 flasks and cultured in growth medium at 37°C under 5% CO_2 until confluent.

2.2.3 Culture expansion

At confluence, cells were trypsinized and either frozen at 1 x 10^6 cells/ml in Freezing Medium (Invitrogen) or passaged using growth medium at 1 x 10^6 cells/T150 flask. MSCs (n=4; mean age, 41±24 years, equal male vs. female, with confirmed colony-forming abilities and tri-lineage differentiation potential[164], Appendix A) were utilized at passage 3-5. MPCs (n=4; mean age of 24±4 years, all male, Appendix A) were utilized at passage 5-8. Human foreskin fibroblasts (HFFs), obtained as a cell line (Lonza), were seeded from frozen vials at 1 x 10^6 cells/T150 tissue culture flask and passaged as above and utilized at passage 6-10. Human dermal ECs were obtained from the Centers for Disease Control and Prevention with Material Transfer Agreement, and cultured with EGM-2MV Bulletkit (Lonza), passaged or frozen as above, and utilized at passage 8-10.

2.2.4 Conditioned medium generation from monolayer cell cultures

Upon reaching ~90% confluence, cells were passaged to a new density of 1 x 10^4 cells/cm^2 in growth medium. After 24 hours, cells were washed once each with PBS and Hank's Balanced Salt Solution (HBSS, GIBCO), and cultured for 72 hours in 0.24 ml/cm^2 basal medium (DMEM, 1% insulin-transferrin-selenium-X [ITS-X], 2% PSF). Non-cell conditioned control CM was generated by incubating basal medium in empty flasks. All CM samples were centrifuged at 200 x g for 5 minutes and the supernatant frozen at -20°C for later use. At the time of use, CM samples were thawed and again centrifuged at 200 x g for 5 minutes before use in culture. To determine the relative amount of protein produced per cell, CM was concentrated 20x using Amicon Ultra-4 centrifugal concentrator columns (3kDa cut-off, Millipore). CM protein content was determined using a BCA Protein Assay (Pierce) and bovine serum albumin standard curve.

2.2.5 siRNA silencing of VEGF

MPCs and MSCs were seeded at 2 x 10^4 cells/cm^2 in 6-well plates (Costar). After 24 hours, VEGF gene expression was targeted for silencing with SMARTpool silencing (si)RNA and Dharmafect1 (Dharmacon) according to the manufacturer's instructions. After an overnight transfection, cells were washed and incubated in FGF-2-free growth medium for 24 hours. This wash procedure was repeated 48 hours post-transfection, and transfected cells were utilized for subsequent experiments beginning 72 hours post-transfection.

Following transfection and washes, cells were lysed with RLT buffer (Qiagen). After RNA was isolated and converted to cDNA (Superscript III: Invitrogen), VEGF165 and β-actin

primers (Qiagen, proprietary sequences) were utilized for qPCR gene expression analysis (SYBR Green: Invitrogen) on AB Hit9000 (AB).

For CM collection, seventy-two hours post-transfection cells were washed in PBS and HBSS and 3 ml of basal medium were added, followed by additional culture for 48 hours. The collected CM aliquots were kept frozen at -20°C until analysis by sandwich ELISA against human VEGF, PDGF, FGF2, and IL-1β (R&D).

2.2.6 *In vitro* angiogenesis: cord formation assay preparation, CM addition and cord maturation

Cord-forming surfaces were prepared by spreading 10 μl ice-cold growth factor-reduced Matrigel (BD)/cm^2 onto 96-well non-tissue culture treated plates (Costar; ibidi). Matrigel was allowed to gel at 37°C for 1 hour, followed by the addition of 100 μl basal medium to the wells. After an additional hour, 90 μl of basal medium was removed from each prepared Matrigel-containing well. 2 x 10^4 ECs in 20 μl basal medium were seeded in each well, and allowed to adhere to Matrigel undisturbed for 1 hour.

A 200 μl aliquot of CM was added to each well. EC cultures were incubated overnight for 16 hours, washed in PBS, and fixed in 2% paraformaldehyde. Bright-field mosaic images of connected cords formed in each well were obtained at 40X magnification using an inverted microscope (Olympus IX81) with automated stage, controlled by Metamorph software. Multiple images (>40) from each well were batch processed with a custom macro to allow analysis with Angiogenesis Analyzer[165] (Fiji/ImageJ). False counts due to clustered cells or contrast created by

well curvature were excluded. Network length and extent of branching per microscope field were compared for all groups.

2.2.7 MPC/MSC/HFF live cell encapsulation in constructs

At ~90% confluence, cells were trypsinized, pelleted, and resuspended in visible light-activated (photoinitiator: 0.1% lithium phenyl-2,4,6-trimethylbenzoylphosphinate) 10% (w/v) methacrylated-gelatin, as described recently[166]. Ten μl of gel were placed on a marked, 5 mm round glass coverslip, and visible light applied for 5 minutes using a commercial illuminator (LEDwholesalers; 450 nm) to create uniform-sized constructs. Pellets were washed to remove the photo-initiator and stored briefly in Phenol Red-free DMEM before placement on CAM or in culture *in vitro*.

2.2.8 Cell proliferation in cell-seeded constructs and collection of conditioned medium

Constructs were cultured in Phenol Red-free DMEM (1 ml/6 constructs) for up to 48 hours. CM from constructs was collected daily and analyzed for angiogenic factors by ELISA, as described above. Construct cell density and viability were assessed by Live-Dead staining (Invitrogen); live and dead cells occupying arbitrarily selected space within the construct volume were quantified using a custom NIH ImageJ macro.

2.2.9 CAM angiogenesis

Induction of vessel recruitment *in vivo* was assessed with the CAM assay, using chick embryos in shell-less culture[167]. Three to six cell-seeded constructs were distributed, 2-3 cm apart, on the surface of each CAM of incubation day 9 chick embryos. After 48 hours, small (<40 μm) blood vessel invasion into the construct was imaged (SZX10 Olympus) and counted by a blinded observer. Constructs were then excised from the CAM, and the number of live cells quantified as described above.

2.2.10 Statistical analysis

At least three independent experiments with at least three replicates per condition were performed for each assay. Outliers were removed with Grubbs' test. All data were presented as mean $\pm$ standard deviation (SD). Significance was determined by Student's t-test (n=3) and one- or two-way ANOVA (n>3), as appropriate, with Tukey's post-hoc analysis (GraphPad Prism).

2.3 RESULTS

2.3.1 MPC effects on EC cord formation in vitro: VEGF involvement

To determine if MPCs contribute, ultimately to blood vessel recruitment, the effect of MPC-CM on EC cord network complexity was assessed. Because MPC-produced VEGF might play a maor role MPC-associated vessel recruitment, MPC- and (control) MSC- VEGF was removed by

siRNA silencing. After confirming that VEGF was effectively silenced, the effect of MPC- and MSC- produced-VEGF on EC network complexity could be determined.

MPCs and MSCs were tested for their ability to influence the cord-forming abilities of Matrigel-plated ECs (Figure 5). Only CM generated by MPCs positively influenced the complexity and length of EC networks relative to control (non-cell-conditioned) CM. VEGF production in MPCs and MSCs was efficiently silenced using SmartPOOL siRNA. Decreases in VEGF gene expression were observed, and CM from gene specific siRNA treated populations contained significantly lower levels of VEGF than control cultures (Figure 6). Interestingly, silencing of VEGF significantly affected the ability of MPCs to stabilize EC networks, but silencing did not appear to affect the angiogenic activities of MSCs (Figure 7). MPC support of EC network formation is particularly impressive given the small but significant difference in measured protein concentrations between the pooled CMs (MPC-CM 2.8 ± 0.5 mg/ml, MSC-CM 3.8 ± 0.7 mg/ml, $p<0.05$).

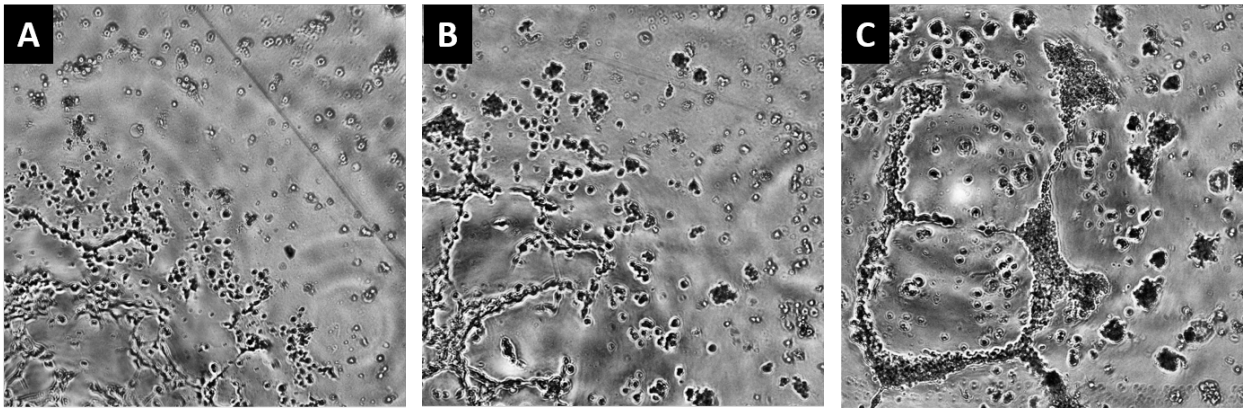

Figure 5: Representative images of EC networks seen *in vitro*
Networks were formed during 16-hour exposure to (A) cell-free control CM, (B) MSC-CM, and (C) MPC-CM. MPC-CM encouraged the creation of longer and more complex EC networks.

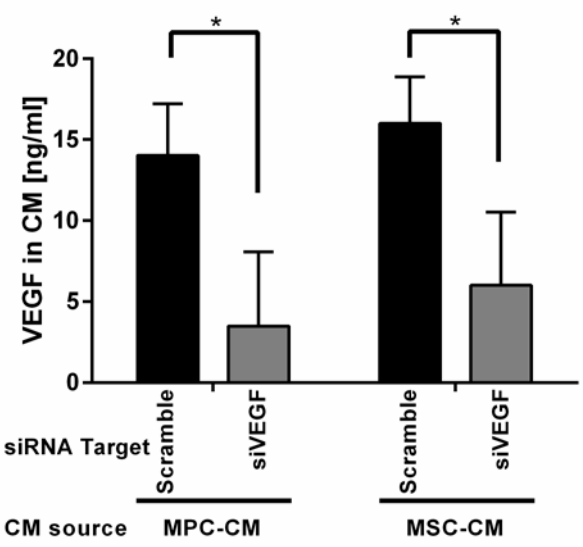

Figure 6: VEGF silencing by siRNA successfully decreased secreted VEGF concentrations
Treatment with sequence-specific VEGF siRNA significantly reduced VEGF concentrations in CM of both MPC and MSC. * $p<0.01$, n=6.

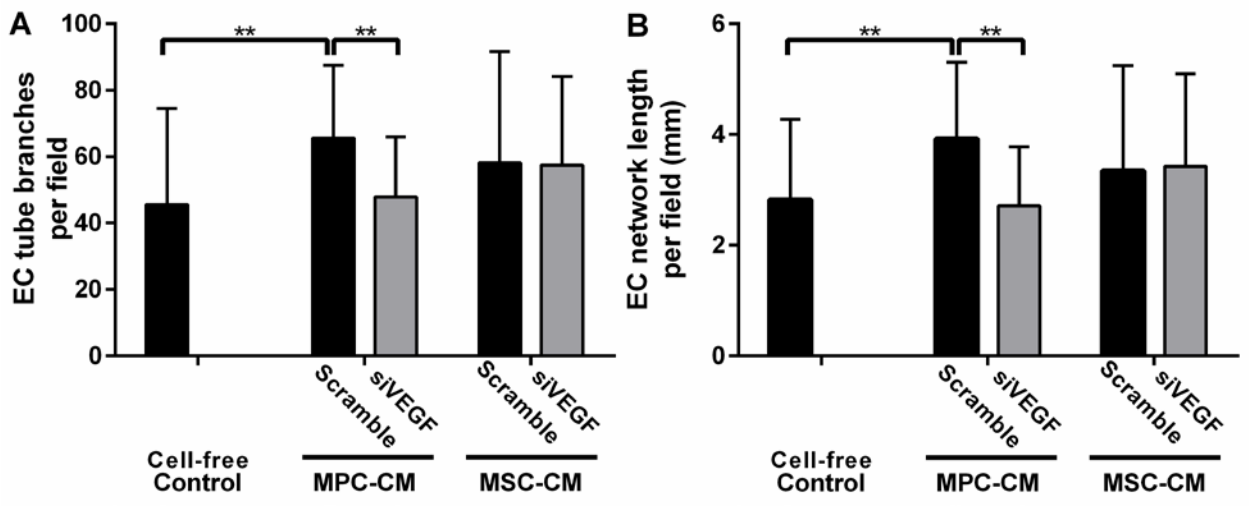

Figure 7: Effect of CM from VEGF-silenced MPCs and MSCs on the organization of EC cord networks, assessed based on (A) EC tube branching, and (B) EC networking
MPC-CM enhanced the cord-forming ability of ECs, and that support was significantly decreased by VEGF silencing; MSC-CM did not significantly affect EC cord-forming abilities regardless of VEGF content.. n=3, **$p<0.01$.

2.3.2 MPC hydrogel construct

The ability of MPCs and MSCs to enhance EC cord formation prompted examination of their pro-angiogenic activities *in vivo* when delivered in cell-seeded scaffold constructs, as a first step to evaluate the therapeutic potential of autologous cell implantation. For such an application, a crucial requirement here was the long-term viability of the seeded cells within the scaffold construct. A photocrosslinked gelatin-based hydrogel recently developed in our laboratory, which exhibited excellent cell retention and compatibility properties[168,169], was therefore used to encapsulate MPCs and MSCs. For this study, MPCs or MSCs were seeded in 10 μl photocrosslinked methacrylated-gelatin at various densities (10×10^3, 50×10^3, and 100×10^3 cells per construct, designated as 10k, 50k, and 100k, respectively). MPC and MSC cell number and viability at 24 and 48 hours after encapsulation were determined by automated cell counting of flattened vertical stacks of Live-Dead stained cells (Figure 8). Given culture under serum-free conditions, both MPCs and MSCs exhibited reasonably high viability (>60%), and cell number did not change significantly for any group over the course of the observation, implying neither pronounced cell death nor proliferation. Very few cells were observed to have migrated from the constructs onto the surrounding tissue culture plastic.

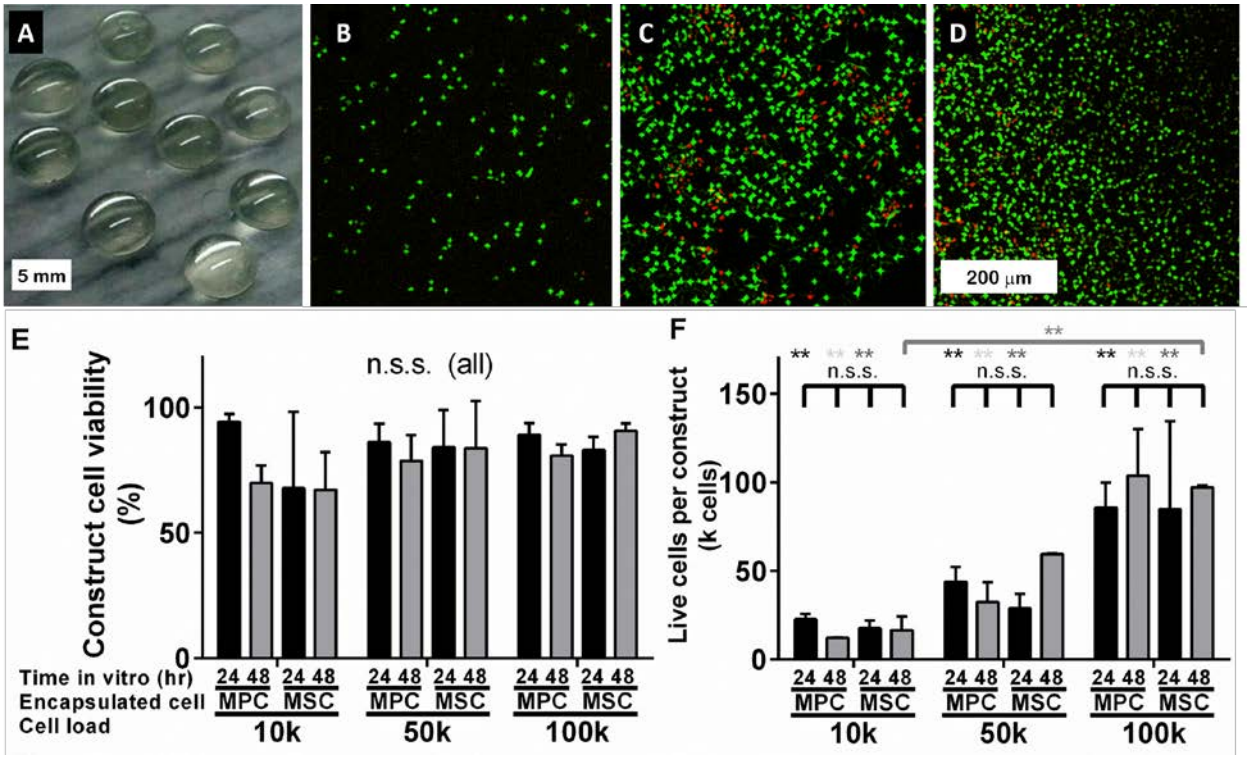

Figure 8: Encapsulation of MSCs and MPCs within photo-crossslinked methacrylated-gelatin constructs

(A) Ten μl photo-crossslinked methacrylated-gelatin constructs encapsulating (B) 10k, (C) 50k, and (D) 100k cells per construct. Scale bars as indicated. Viability (E) and number (F) of encapsulated cells determined by Live-Dead staining. Stained cells were imaged in vertical stacks that were processed and used to determine construct viability and abundance after 24 and 48 hours in serum-free DMEM. Cell viability remained high (>60%) with no evidence of marked cell death or proliferation without regard to cell type or days in culture. $n \geq 3$, $^{**}p < 0.01$ due to cell loading differences at the same timepoint and loading density. n.s.s. = not statistically significant.

2.3.3 Cytokine release from constructs

Because hydrogels are often used to sustain the release of therapeutic growth factors *in vivo*[74], it was important to assess the level of growth factors secreted by the cells into the conditioned medium. CM generated in the above serum-free experiment was collected and assayed for the presence of VEGF, showing time-dependent release profiles (Figure 9). Initial experiment also

showed that FGF-2 was detected at ~100 pg/ml in CM derived from two MPC and one MSC patients, but subsequent results were variable. PDGF and IL-1β were not detected in the CM. As a positive control, cell-free constructs encapsulating only the tested growth factors released >80% of the encapsulated growth factor after 24 hours (Appendix B), implying minimal retention of cell-secreted growth factors by the biomaterial scaffold.

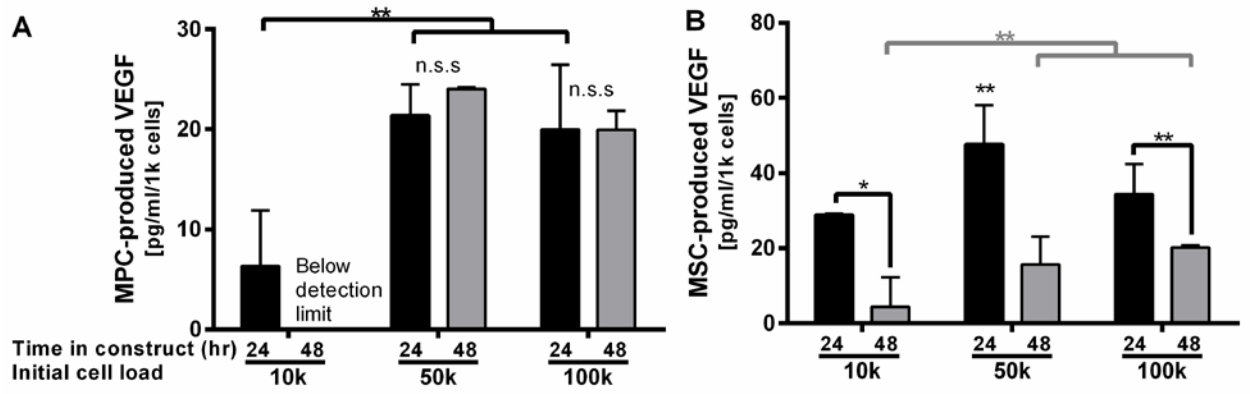

Figure 9: Production and release of VEGF by (A) MPCs and (B) MSCs encapsulated in photo-crosslinked constructs
VEGF was produced as a function of initial cell seeding density with 50k initial cells/construct as the most efficient. The results showed MPC-VEGF production was unaffected by time in culture, in contrast to MSCs. n=3; *p<0.05, **p<0.01. n.s.s. = not statistically significant.

2.3.4 *In vivo* angiogeneic activity of cell-seeded constructs using the CAM assay

Forty-eight hours after the MPC- and MSC-encapsulated gelatin constructs were placed on the CAM surface, the number of capillaries recruited to the construct was quantified by a blinded observer (Figure 10A-E). The results clearly showed the pro-angiogenic activity of both MPCs and MSCs, with the former being more potent, particularly at higher cell seeding density. In comparison, a control cell type, the HFFs, was completely inactive at all seeding densities.

The potential involvement of VEGF in mediating capillary recruitment was also investigated in the CAM system by utilizing the VEGF-silenced cells described earlier (Figure 6), seeded at 50k cells/construct (n=2); however, no significant differences in capillary recruitment were apparent between treatment group or cell type (Figure 11), possibly due to excessive endogenous CAM VEGF production. Experiments with adult animals might show heightened VEGF responsiveness.

After 48 hours, constructs were removed from the CAM, and viable cells within the construct were quantified with Live-Dead assay as described above (Figure 8). Cell-free constructs were similarly examined to correct for non-specific cell migration from the chick CAM, and the maximum number of adherent/invasive cells subtracted from the reported values (typically on the order of 10-100 adherent cells per construct). The results showed that, as was the case for constructs maintained *in vitro* (Figure 10F), significant maintenance of viable cells, including at the highest density of 100k. This finding suggested that MPCs implanted in a hydrogel construct could remain viable and active for longer durations and that additional testing using this approach of MPC-based therapy should be applicable in larger animal models and for longer duration.

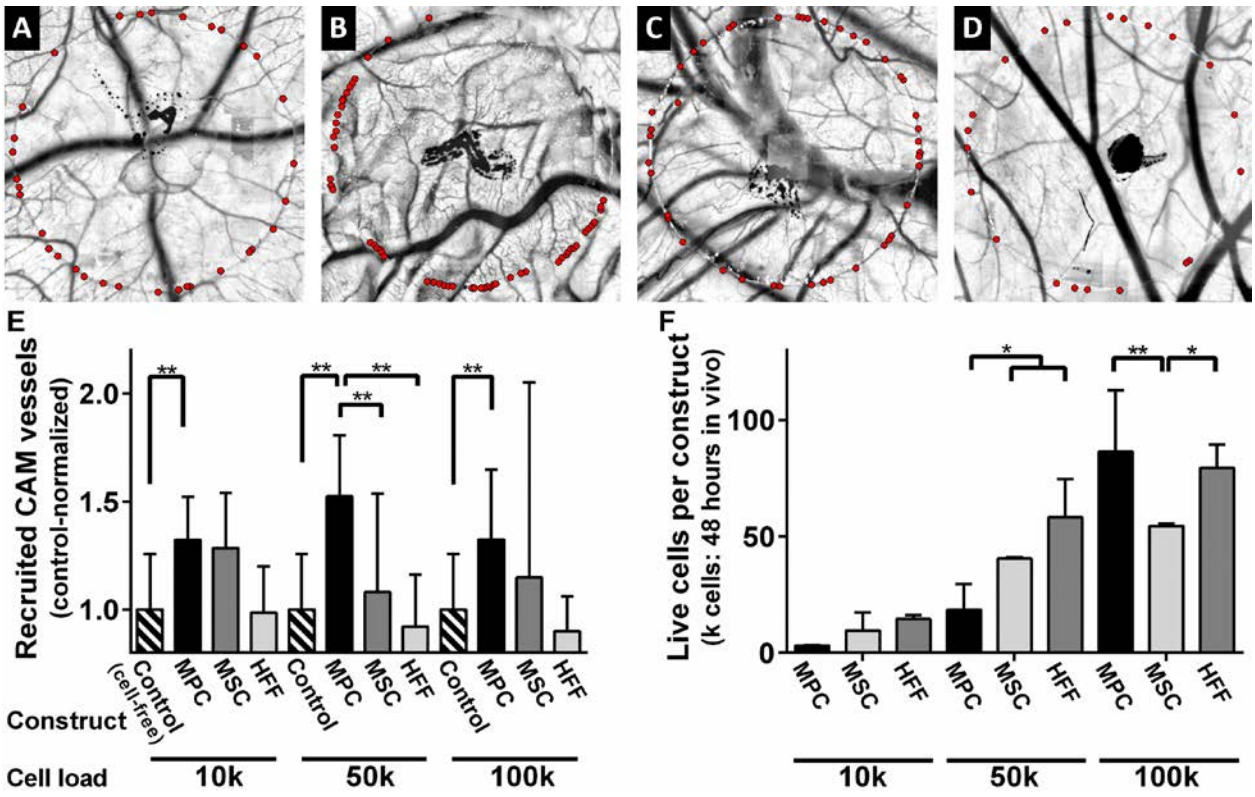

Figure 10: *In vivo* CAM assay of pro-angiogenic activity of MPCs
Cells (MPCs, MSCs, and HFFs) were encapsulated in photocrosslinked gelatin hydrogel, and the constructs placed on day-9 chick embryonic CAM. Forty eight hours after implantation, angiogenic induction in (A) cell-free control, (B) MPC, (C) MSC, and (D) HFF groups was assessed by counting the (E) number of CAM capillaries recruited to each construct using contrast-enhanced dissecting microscope images; $n \geq 8$, $^{**}p<0.01$. Black marks near the center of each coverslip (A-D) allowed easy identification of constructs on the CAM surface. Small vessels, denoted by red circles, were counted if they appeared to cross the edge of the 5mm diameter coverslip (A-D). Representative images for each encapsulated cell type (B-D) at an initial seeding density of 50k cells/construct were shown. (F) Encapsulated cell viability was assessed via Live-Dead staining; $n \geq 3$, $^{*}p<0.05$, $^{**}p<0.01$. MPCs recruited significantly more vessels than any other cell type, and despite fewer surviving encapsulated cells by 48 hours, MPCs recruited blood vessels more efficiently than any other cell type at an initial cell density of 50k cells/construct.

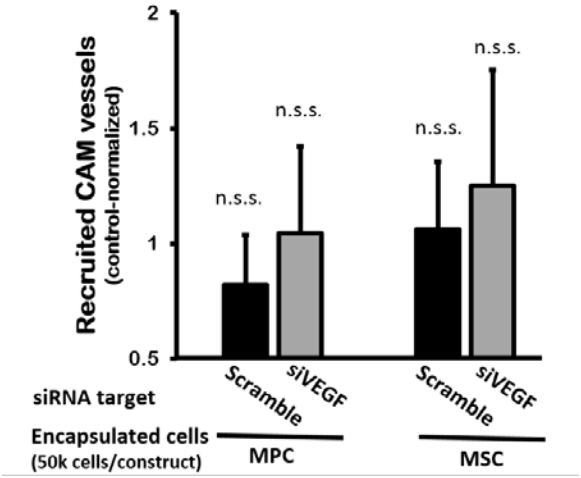

Figure 11: *In vivo* CAM assay of pro-angiogenic activity of VEGF-silenced MPCs and MSCs
The number of CAM vessels recruited by VEGF silenced MPCs and MSCs was not significantly different from control groups, indicating that exogenous VEGF in the context of embryonic vessel recruitment is insufficient to markedly alter capillary recruitment; n = 3.

2.4 DISCUSSION AND CONCLUSIONS

The present work confirmed gene expression-regulated production of VEGF (Figure 6) by MPCs and MSCs via sandwich ELISA analysis of CM, while production of IL-1β, FGF-2, and PDGF by both cell types was inconsistent or undetectable. The MPC secretome was observed to have a net positive effect on the organization of EC cords *in vitro*, and this effect was abrogated by siRNA silencing of VEGF. MSC-secreted VEGF did not alter the net-zero effect of MSC-CM on EC microvascular network length and extent of branching (Figure 7). To test the suitability of MPCs for use *in vivo*, MPCs were encapsulated and cultured in vitro within a mechanically-tunable, photocrosslinked hydrogel scaffold[169] (Figure 8). A majority of the encapsulated MPCs survived and continued to produce VEGF, and while MSCs tolerated

encapsulation, MSC-VEGF production declined over time (Figure 9). After two days on the surface of the CAM, most encapsulated cells were still viable and relatively few CAM cells had migrated into each construct. Consistent with the *in vitro* assays, MPCs exhibited more efficient capillary recruitment abilities than MSCs (Figure 10), but that activity was no longer VEGF dependent. Non-specific cell- or hydrogel-mediated CAM inflammation was ruled out due to the observed lack of CAM response to encapsulated HFFs and normalization to cell-free control.

VEGF is a potent and highly-expressed angiogenic molecule that acts to affect endothelial cell migration, proliferation, and tube formation, among other functions[49]. When coordinated with other molecules, VEGF-associated EC function changes result in angiogenesis, the formation of new vessels as extensions of existing vasculature. Besides VEGF, serum-starved or otherwise-activated MSCs produce a host of molecules that can modify or mask the effect of VEGF on angiogenesis and wound healing, including ANGPT-1, FGF-2, IGF-1, HGF, SDF-1, and PDGF[147,151,156,170], that variously increase EC proliferation, migration, branching, and/or adhesion. Additionally, cytokines that affect both endothelial cells and surrounding inflammatory cells, including CCL-2, IL-1β, and IL-6 can aid in the loosening of EC-EC connections, allowing EC tip cells to sprout and form nascent blood vessels[90,155,171,172]. However, MSC-secreted molecules are not purely pro-angiogenic. Anti-angiogenic activity in MSCs has been observed to be mediated through several routes, including through soluble, decoy forms of growth factor receptors such as soluble PDGF receptor[173], soluble VEGF receptors (sVEGFR-1 and sVEGFR-2)[35] and through exosomes/microparticles isolated from MSC-CM, some of which contain VEGF-inhibiting microRNA[174–176]. While less thoroughly-characterized than MSCs, several of the above-mentioned pro-angiogenic factors are known to be expressed or produced by MPCs. Similar gene expression levels for IL-1β, IL-6, VEGF, and FGF-2 were observed between MPCs

and MSCs[139]. Comparative CM analysis could elucidate interesting differences that might explain the observed differences in EC support activities between MPCs and MSCs.

One well-established in vitro model for angiogenesis involves the seeding of ECs on growth factor-reduced Matrigel or similar laminin-rich substrate and observing EC network characteristics; the cord-forming assay as it was implemented in this work allowed a complete description of EC networks formed within an entire well. Other in vitro angiogenesis models required manual selection of imaging areas (3D tubule assay), leading to possible experimental bias, or assayed the response of improperly sourced (fetal- or aortic-) endothelial cells[177]. EC cord-networks are extremely dynamic, maintaining highly-branched multi-cellular configurations under the influence of pro-angiogenic factors and regressing to relatively inert cell clusters within a few hours following the removal of pro-angiogenic factors. Because the ECs used here were derived from a stably-transformed microvascular cell line[178], and cord-formation versus physiologically functional vascular tube formation was assessed, it is possible that the pro-angiogenic effects of MPCs observed *in vitro* will not transfer to an in vivo setting.

To relate *in vitro* and *in vivo* settings, various *in vivo* angiogenesis models exist; however, each *in vivo* model comes with its own limitations. Of high priority is the cost to both the researcher and animal, as an entire, possibly immunocompromised animal must be bred, housed safely for the duration of the experiment, and sacrificed to allow analysis of a relatively small tested area (rat or mouse skin pocket, windowed rabbit ear, or corneal micropocket)[177]. Although limited to the short time period of embryonic development, the shell-less chick CAM assay offers the advantage of a relatively large and easily-imaged angiogenic surface, allowing for up to six assays per embryo, and several dozen assays can be housed in a standard static incubator. The relatively short assay, occurring over a matter of days, minimizes any suffering of the

developing embryo, but appropriate controls must be utilized on every CAM as local tissue inflammatory responses are highly variable from embryo to embryo. While the argument can be made that observations of avian and human cell interactions are not likely to be relevant to human wound healing, the widely-used CAM assay offers a useful and relatively inexpensive screening tool to identify populations of broadly pro-angiogenic cells, or specific factors produced by cells, that have a high likelihood of promoting angiogenesis *in vivo*. With the CAM assay, we observed marked vessel recruitment by MPCs with significant and strong recruitment at median cell density. Work is currently underway to confirm MPC therapeutic activities in a mammalian system.

Given some reports on the high dependence of MSC-angiogenic activity on VEGF, as presented in the introduction[162], our finding of minimal pro-angiogenesis by MSCs should be considered in view of a recent study on the source-related differences in MSC angiogenic function, which showed similar, underwhelming bone marrow-derived MSC support of *in vitro* angiogenesis when compared to adipose-derived MSCs[179]. This cell source-related difference in angiogenic potential both *in vitro* and *in vivo* could be related to different mechanisms utilized by each cell type to promote angiogenesis, as has been reported for adipose-derived versus bone marrow MSCs[180,181]. Future work analyzing and comparing the secretomes and matrix interactions of MPCs and variously-sourced MSCs is critical in determining specific applications where MPCs might better meet therapeutic challenges than clinically-available MSCs.

A few caveats can be applied to the above conclusions. As has been identified in previous studies, the MPCs studied here were derived from a fairly homogeneous young, male donor population compared to the older, mixed gender MSC donor population. Given comparable VEGF production and proliferation rates between the two populations, it is unlikely that age-

related changes account for the observed differences[123]. Additionally, this work has presented ECs and the capillaries that they form as relatively passive interpreters of MPC- or MSC-angiogenic signals; in reality, ECs themselves can regulate angiogenesis via several mechanisms, such as altered Tie-2 expression and/or ANGPT-2 release[154,182]. The nature and regulation of interactions between ECs and MPCs must be elucidated to better manipulate the ultimate therapeutic potential of MPCs.

Taken together, these findings suggest that MPCs preferentially promote angiogenesis in a manner distinct from and possibly superior to MSCs. This effect is likely due to a combination of MPC-secreted VEGF activity on ECs and other, possibly matrix-modifying factors[183,184], affecting endothelial, endothelial cell-associated, or immune cell activities. MPCs are therefore likely to be useful in the recruitment and stabilization of vasculature necessary to support an implanted construct. The significant advantage of their easy isolation and autologous sourcing further lends itself to the potential therapeutic use of MPCs. If encapsulated within a degradable hydrogel, viable and autologous MPCs could conceivably be used to procedures that require enhanced vascular support, including soft tissue or bone reconstruction in blast-traumatized limbs.

3.0 NEUROTROPHIC SUPPORT BY MPC AND EC: A ROLE FOR VEGF?

3.1 INTRODUCTION

The capacity for nerve regeneration after injury is related to the mechanism of injury and the time to treatment. While autograft nerve and blood vessel transplantation are the clinical treatments of choice, donation sites of appropriate size are limited and require a second surgery, damaging the otherwise healthy tissue of an already taxed patient. Severely-injured patients can be treated with synthetic, biodegradable nerve guides as a last resort, but such guides are associated with less favorable outcomes[51].

The most sophisticated nerve guide conduits attempt to mimic the physical, chemical, and temporal aspects of the native repair processes. For synthetic guide-scaffolds, substrate patterning or the incorporation of aligned fibers mimic bands of Büngner formed by proliferating Schwann cells[185,186]. Advancements in polymer chemistry and refinements of electrospinning technique allow the precise control of the fiber diameter, alignment, and degradation rate [187,188] with the ultimate goal of complete scaffold replacement by native tissue. Poly-caprolactone (PCL) is a biodegradable synthetic polymer that can be easily electrospun; the *in vivo* degradation rate is comparable to the rate of native tissue replacement[76]. Additionally, PCL nerve guide conduits are clinically-approved, lowering the potential regulatory barriers to use[60].

In addition to physical guidance, the maintenance of Schwann cell-secreted neurotrophic factors is important for motor and sensory nerve survival and extension towards the target tissue[67]. These secreted factors include, but are not limited to, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and glial cell derived neurotrophic factor (GDNF), and they can be tethered to or encapsulated within the nerve guide conduits for controlled release[65,186,189].

The bioactivity of exogenously-delivered factors may degrade with time, and experiments have shown that direct transplantation of dynamically-responsive cellular growth factor sources, such as Schwann cells and Schwann cell-like bone marrow-derived mesenchymal stem cells (MSCs), can enhance nerve repair outcomes[190,191]. Nerve co-culture with or treatment with conditioned medium derived from Schwann cells, Schwann like-MSCs, or undifferentiated MSCs positively influenced the length and density of neurons dissociated from embryonic dorsal root ganglia (DRG) [192,193].

In addition to stimulating the damaged nerve, MSCs are thought to signal the surrounding support tissue and (possibly) invading immune cells; the net result of MSC activity is a positive effect on nerve defect healing[157,194]. The use of allogeneic MSCs, while clinically approved for certain therapies, face intensifying scrutiny over their immunogenicity[111]. Currently, the sourcing of autologous MSCs, even the minimal procedure required for adipose-derived MSCs, also involves invasive procedures.

Recent studies have shown that surgical waste, obtained following standard debridement of traumatic extremity injuries, yields a population of blast-traumatzied muscle-derived multipotent progenitor cells (MPCs) [5]; MPCs are poised to be ideal candidates for nerve repair. MPC activities are similar to those of widely-known and clinically-utilized bone marrow-derived

MSCs[163]. MPCs express NGF, BDNF, CNTF, and neurotrophin(NT)-3[141]. MPC culture in a defined medium for neurotrophic induction enhances the expression of these factors[141] and stimulates neurite outgrowth in embryonic dorsal root ganglia [140]. MPCs also exhibit the ability to control their local proteolytic microenvironment [160], suggesting that MPCs might also contribute neurotrophic benefits through indirect means.

Vascular cells, particularly endothelial cells, are intimately connected with the development, growth, and continued health of nerves[191], often utilizing similar molecular pathways for physical guidance[29]. MSCs are associated with endothelial cells and may interact with them in the maintenance of the stem cell niche and in endogenous regenerative processes[105,183,184]. There is increasing evidence that the endothelial cell ligands vascular endothelial growth factor-A (VEGF) and angiopoietin (ANGPT)-1 also exert effects on neurons, especially in the context of stroke in the central nervous system[195]. Although VEGF receptors (VEGFR) are expressed by neurons[196], the role of VEGF signaling in regeneration is not defined. Recent clinical studies have reported on VEGF promoting anatomical and functional recovery of injured peripheral nerves in the avascular cornea[197].

Extrapolating from these findings, we hypothesize that MPCs positively modulate nerve growth activities and that this effect could be augmented through interactions with endothelial. These interactions were investigated using the embryonic DRG model in two experimental set-ups: (1) treatment of DRGs cultured on tissue culture plastic with one or more cell-conditioned media and (2) co-culturing of DRGs and MPCs seeded onto a nanofibrous biomaterial nerve conduit scaffold. The results showed that neurotrophic differentiation of MPC more strongly affected DRG neurite extension on nanofibers, regardless of the presence or absence of endothelial cells. Nanofiber-seeding of MPCs increased the gene expression but ultimately

resulted in lower conditioned medium (CM) concentrations of several neurotrophic factors. CM investigations revealed that VEGF or VEGF-associated molecules in MPC-CM, cultured under neurotrophic or growth conditions, were responsible for a large portion of the observed neurite length enhancement effect. In comparison, the neurotrophic support activity of MSC-CM was largely unaffected by VEGF removal. This observation suggests important differences between the neurotrophic secretome of MPCs and MSCs.

3.2 METHODS

3.2.1 Construct formation

Aligned nanofiber constructs (NFCs) were deposited reproducibly on glass slides by electrospinning, at ~15 kV difference from a 22G needle placed 15 cm from a rotating mandrel, an 11.5% poly-ε-caprolactone (PCL) solution dissolved in 1:1 tetrahydrofuran:dimethylformamide. NFCs were completed by lining each nanofiber-coated slide with silicone glue. NFCs were dessicated overnight to remove any residual solvent, sterilized with ethanol and UV-light exposure, and rinsed with PBS before incubation with either 10% fetal bovine serum (FBS: Invitrogen) or, serially, with 100 ng/ml polylysine- and 10 μg/ml laminin-coating (Sigma-Aldrich: 4°C, overnight). Fiber thickness and orientation were determined using scanning electron microscopy and plugins developed for ImageJ/Fiji (BoneJ 1.4.0[198]; Directionality 2.0[199]).

3.2.2 Nerve DRG culture

DRGs, obtained from incubation day 9 chicken embryos via microdissection, were placed on sterile, aligned polylysine-and-laminin-coated NFCs. After 24 hours in nerve growth medium (Basic Eagle's Medium, 10% horse serum, 1 nM GlutaMax, Invitrogen) supplemented with NGF+ epidermal growth factor (EGF)+ platelet-derived growth factor (PDGF) (Sigma), DRGs with or without human co-cultures were cultured for 4 additional days in a combination medium of 1:1 conditioned medium:DRG basal medium. Medium was exchanged daily. DRGs were fixed and stained immunohistochemically for heavy neurofilament (NEFH) (Sigma; Secondary Alexa-Fluor antibodies, Invitrogen). DRGs were visualized with an Olympus inverted microscope equipped with a motorized stage controlled through MetaMorph. Resultant mosaic images were stitched using Grid/Collection Stitching (Fiji)[200]; the 10 longest NEFH-positive neurite extensions were measured from the geometric center of the original DRG cluster.

3.2.3 MPC and EC culture

Traumatized muscle multipotent-progenitor cells (MPCs), isolated as described previously[163], (4 male patients, average age: 24, passage 5-8) were expanded in tissue culture-treated flasks (Nunc; Fisher) in growth medium (GM), α–minimum essential medium (MEM) + 10% fetal bovine serum (FBS) + antibiotic-antimycotic (PSF) + 1 ng/ml FGF-2 (Invitrogen). Human dermal microvascular endothelial cells (ECs) were cultured on tissue culture-treated flasks in EGM-2MV medium (Lonza). At 90% confluency, cells were trypsinized (Invitrogen) and passaged at 1 x 10^6/T150 flask. All cells were cultured at 37°C with 5% CO_2.

3.2.4 Neurotrophic induction

Cells were seeded at 1 x 10^3 cells/cm^2 on tissue culture plastic (TCP) or NFCs and differentiated neurotrophically using a modified 10-day protocol[140,141,201]. 24 hours after seeding, cells were incubated with αMEM + 10% FBS + PSF supplemented with: 10 mM β-mercaptoethanol (BME: Sigma) for 24 hours and 10 mM βME + 35 ng/ml retinoic acid (RA: Sigma) for an additional 48 hours; for the six following days, cells were incubated with neurotrophic medium (NM; DMEM/Ham's F12 + PSF (Invitrogen) supplemented with 2% FBS, 2% B-27 (Invitrogen), 6 mg/ml RA, 1 ng/ml FGF-2 (Sigma), 10 ng/ml PDGF (Sigma), 150 ng/ml heregulin (an isoform of neuregulin-1) (Sigma), and 10 μM forskolin (Sigma)).

Following neurotrophic differentiation, cells were washed and either lysed with TRiZol or incubated with basal medium (DMEM + 1% ITSX + 1% PS) for 48 hours to produce conditioned medium (CM). CM was centrifuged at 200xg and frozen at -80°C. For DRG co-culture experiments, tissue culture plastic (P)-cultured NM-MPCs were trypsinized and transferred to DRG-containing fibers at a concentration of 1 x 10^3 cells/cm^2.

3.2.5 EC/MPC culture on nanofibrous constructs

MPC and EC were seeded on FBS-coated NFCs at various densities in cell-specific growth medium; medium was exchanged every two days. Cell viability and density were assessed by quantifying Live-Dead fluorescence microscopy images using custom macros written for ImageJ/Fiji.

3.2.6 Confirmation of neurotrophic induction

Neurotrophic factor production/secretion into CM was assessed via sandwich ELISAs (R&D). RNA was isolated with TRiZol for neural gene expression analysis via RT- followed by q-PCR (Superscript III: Invitrogen; 18S rRNA, BDNF, CNTF, GDNF, Nestin, NGF, VEGF: Qiagen).

3.2.7 Selective VEGF removal

VEGF was selectively removed from thawed, centrifuged, and filtered CM using neutralizing antibodies (mouse IgG, mouse aVEGF; R&D) bound to SpinTrap Protein G beads (Sigma). Beads and any bound antibodies/factors were removed from CM by centrifugation at 200xg for 5 minutes. Bound VEGF was eluted from the beads, and selective binding was confirmed via VEGF ELISA (R&D).

3.2.8 Statistical analysis

All data were presented as mean ± standard deviation, unless noted. Statistical differences and p-values were determined by one- or two-way ANOVA and Sidak's or Tukey's test, as appropriate.

3.3 RESULTS

3.3.1 Exploring MPC- and EC- neurotrophic support

On TCP, pooled CM derived from P-seeded NM-MPCs (Figure 12) and GM-ECs slightly increased DRG neurite extension when mixed 1:1 with basal DRG medium. By contrast, CM from NM-MPC:EC-GM mixed with DRG medium (0.5:0.5:1) increased DRG neurite extension length to almost 2x that of control DRG. This finding suggested that a combination of MPC- and EC- neurotrophic activities might better support neurite extension on a nerve guide conduit.

3.3.2 Scaffold fabrication

Efficient nanofiber-based physical guidance of neurite outgrowth requires the presence of appropriately-sized parallel fibers; electrospinning notoriously involves low batch-to-batch consistency[202]. Because multiple batches of NFCs were required for this study, batch-to-batch consistency of fiber diameter and orientation were assessed by examining randomly-selected scaffolds by scanning electron microscopy. Scaffold nanofibers (average diameter 580 ± 280 nm) were relatively well-aligned (22± 17^{o} dispersion).

3.3.3 Cell viability of MPCs and ECs seeded on NFC scaffolds

High density, long-term culture can result in very high levels of oxidative stress; because nerve cultures are very sensitive to oxidative stress[203,204], it was necessary to determine the density of cells that could be maintained long-term on NFCs. MPCs or ECs were initially seeded at varying

densities (0.5, 1, 5, 10 x 10^3 [k] cells/cm^2) on 10 cm^2 serum-coated NFCs. Cell density and viability were assessed daily for the first three days and after an additional week in culture (Figure 13), corresponding to the schedule of neurotrophic induction. Cells were cultured in their respective growth media, to allow for maximum proliferation.

MPC tolerated NFC culture fairly well, reaching an equilibrium density of approximately 1-5k cells/cm^2, but EC coverage and viability dropped steadily over the culture period. Cell aggregation contributed to large variations in within- and between- sample viability and density measurements.

Because of these findings, subsequent experiments involving NFC scaffolds were determined after short (<5 day) cultures and moderate (1k/cm^2) cell seeding densities. For longer term (>5 days) effects on neurite extension and neurotrophic differentiation, ECs and MPCs were cultured on TCP.

3.3.4 Influence of MPCs and EC co-culture on DRG neurite extension

To assess the potential neurotrophic effect of the combination of MPCs and ECs in the context of a nerve guide, DRG were isolated and seeded onto NFC scaffold. To create co-cultures, NM-MPC, GM-MPCs and/or GM-ECs were added at a final density of 1x10^3 (1k) cells per cm^2 24 hours after DRG-seeding. EC-MPC co-cultures were seeded 1:1. NEFH-positive DRG-neurite extensions were assessed after 4 days of co-culture with daily medium changes (Figure 14).

The presence of MPCs, regardless of pre-differentiation, increased the observed length of DRG neurites. Specifically, NM-MPCs increased the observed neurite length to ~3-to-4-fold above the extension lengths of the NFC-seeded DRG alone, confirming the functional utility of

the neurotrophic differentiation protocol. The additional presence of ECs caused a slight increase in neurite length on the NFC scaffold.

3.3.5 Scaffold effects on neurotrophic activities

To assess whether NFC-seeding might have altered NM-MPC-neurotrophic functions, NM-MPC were seeded in induction medium onto NFC and TCP at densities of 1k/cm^2. Neurotrophic gene expression of NFC-seeded NM-MPC did not change significantly compared to TCP-seeded NM-MPC except for an increase in VEGF expression (Figure 15 A and B). However, pooled CM from NFC-seeded NM-MPC exhibited much lower concentrations of cytokines. FGF-2 (130 ± 220 pg/ml) and GDNF (30 ± 50 pg/ml) were detected inconsistently in CM derived from TCP-NM-MPC and were not detected in CM derived from NFC-cultured NM-MPC. CNTF and NGF could not be detected in any samples.

Similarly, EC culture on scaffolds in EC growth medium resulted in significant changes in neurotrophic gene expression (Figure 15 C and D). Only BDNF and VEGF could be detected in CM, and NFC-culture severely reduced the amount of neurotrophic growth factors measured in EC-CM, perhaps due to protein-scaffold interactions[205]. This likely explains the diminished support observed upon MPC- or EC- co-culture with DRGs versus TCP-derived CM-neurotrophic support.

3.3.6 Mechanism of action of neurotrophic MPCs

VEGF, most commonly known for its role in angiogenesis, is expressed robustly by MPCs and, as was shown, secreted in copious amounts upon neurotrophic induction (Figure 15 B

and D). The magnitude of secreted VEGF relative to other neurotrophic factors as well as the observation of VEGFR[206] on the surface of nerves suggested that it might play a stronger in MPC-mediated neurotrophic activity than previously realized. To test whether VEGF was acting as a mediator of the MPC effect on DRG, anti-VEGF antibodies-bound to Protein G beads were used to selectively deplete VEGF from the CM from MPC and EC. VEGF depletion was confirmed by ELISA (Figure 16A). VEGF concentrations in control and IgG-treated medium were identical (~170 pg/ml), and VEGF concentrations in anti-VEGF samples were below the detection limit. VEGF-removal from EC-GM-CM and MPC-CM significantly decreased the maximum length of TCP-cultured DRG neurite extensions (Figure 16B), suggesting a dependence of the neurotrophic activity of both cell types on VEGF in addition to other MPC-secreted neurotrophic compounds.

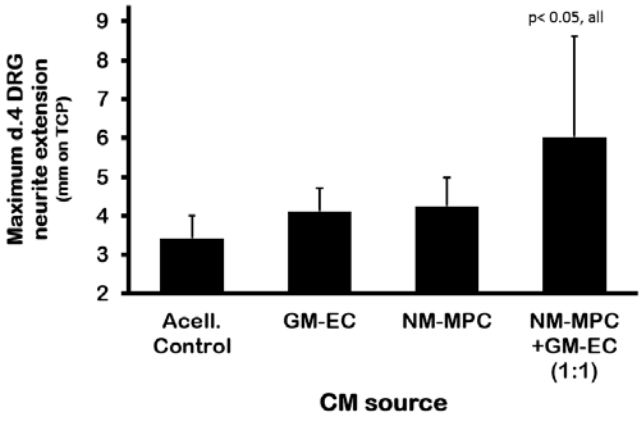

Figure 12: Neurite extension of TCP-seeded DRGs cultured with CM from MPCs and/or ECs

DRG neurite extension increased due to the synergistic effect of NM-MPC-CM and GM-EC-CM; n = 6.

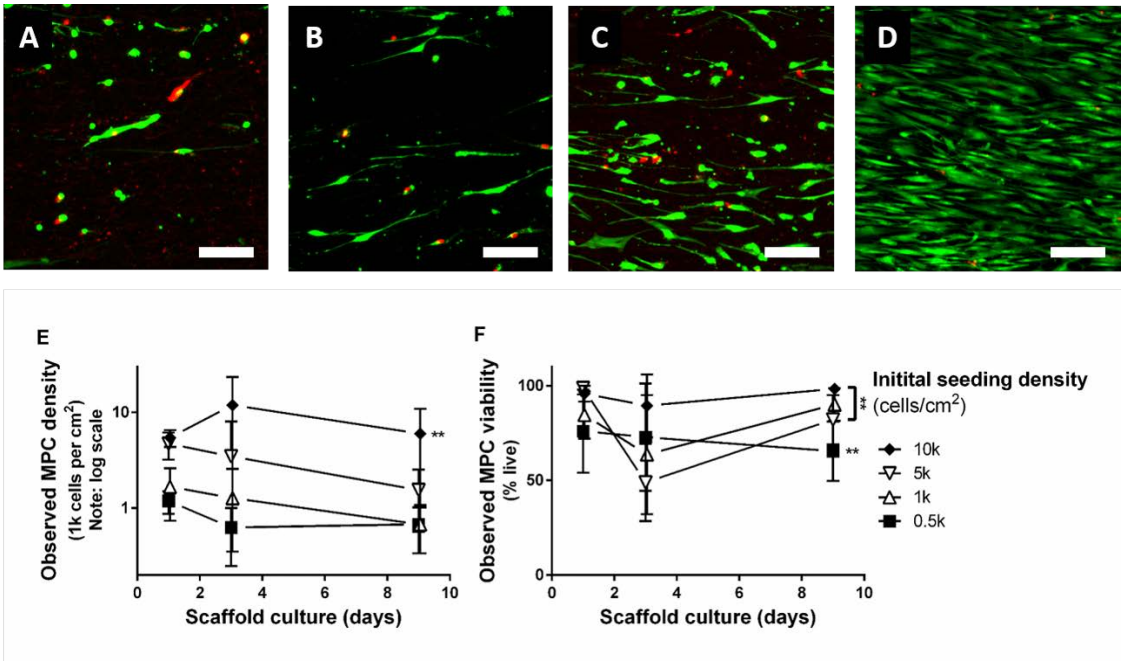

Figure 13: MPC morphology seeded on NFC scaffold

Edge-to-edge mosaic images of MPCs stained with Live-Dead assay were obtained at 24 hours post-seeding at (A) 0.5k (B), 1k, (C) 5k, and (D) 10k cells/cm^2; scale bar = 100 μm. Evidence of NFC alignment could be seen in the common orientation of the MPCs. (E) MPC density scaled with initial seeding density at early timepoints (days 1 and 3), but eventually converged towards 1k cells/cm^2 by day 9. Because of MPC aggregation on the scaffolds, (F) MPC viability varied widely at low timepoints; while still high (>60%), some variability remained by day 9.

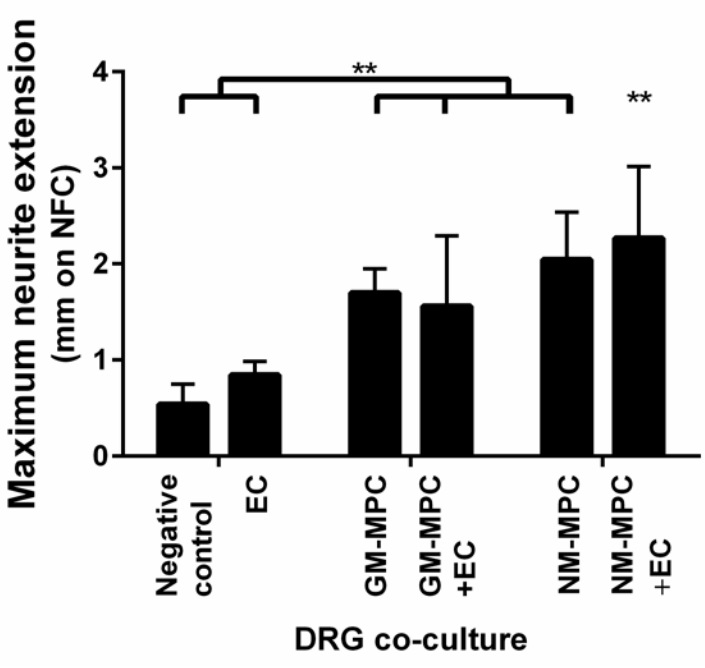

Figure 14: Neurite extension of DRG seeded on NFC scaffold upon co-culture with MPCs or combination of MPCs/ECs (1:1)

MPC influence, particularly neurotrophic induction, increased the length of neurite extensions. With respect to each treatment group, EC presence did not significantly alter the length of observed neurite extensions except in co-culture with NM-MPC.

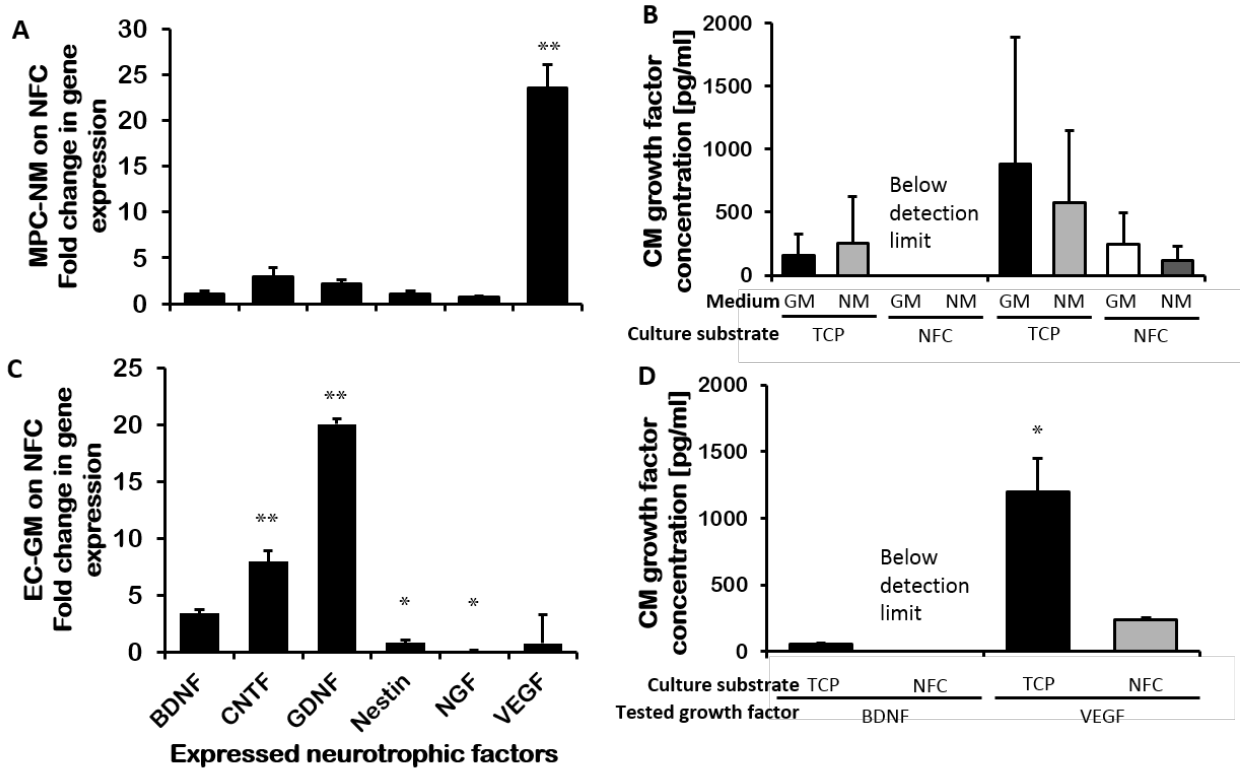

Figure 15: Culture substrate effects on MPC cytokine gene expression
MPC (A,B) and EC (C,D) cytokine gene expression (A,C), normalized to TCP-controls were mildly or positively affected by NFC-culture in almost all cases. Cytokine secretion, however, (B,D) was adversely affected by culture on NFC. n = 6. * $p < 0.05$, ** $p < 0.01$ from all other groups.

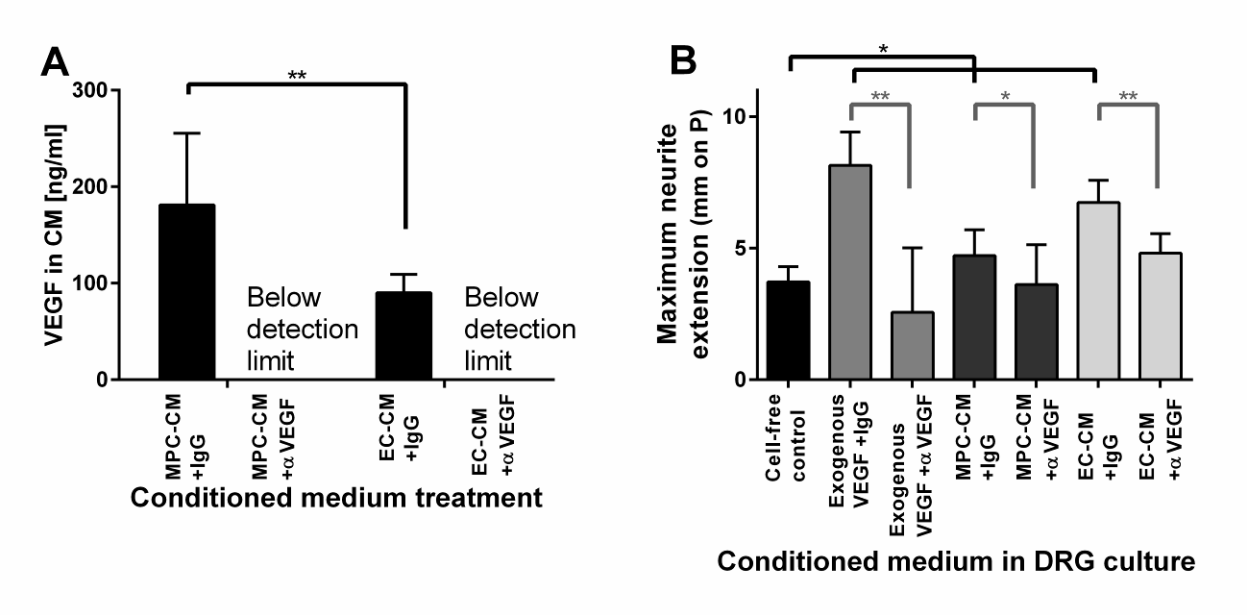

Figure 16: VEGF production by MPCs and MSCs and effect on neurite extension
VEGF was present in conditioned medium derived from both MPCs and MSCs (A) and could be removed P-CM by indirect immunoprecipitation using VEGF-specific antibodies (versus control IgG antibodies). Selective removal of VEGF from control (exogenous VEGF-only) and 1:1 DRG medium:cell-conditioned medium groups decreased observed neurite extensions compared to IgG-treated mixtures, indicating that VEGF contributes to both MSC and MPC neurotrophic function. n=3, **$p<0.01$.

3.4 DISCUSSION

We have previously reported that that neurotrophic induction of MPC enhances their production of neurotrophic factors, most notably BDNF and CNTF. Extending that work, results from this study showed that NM-MPC co-culture and CM from NM-MPC supported neurite extension of DRGs seeded on NFC and TCP, respectively, despite adverse effects of the nanofibrous substrate on MPC viability and growth factor secretion into the CM. While consistent, significant differences in VEGF or BDNF concentrations in the CM were not observed due to high between-patient and between-experiment variability, distinctly higher VEGF concentrations were associated with neurotrophic induction.

Our findings showed that the combination of NM-MPCs and GM-EC might best support neurite extension (Figure 12), working almost synergistically despite similar concentrations of BDNF and VEGF in their respective CM (Figure 15 B, D). Other researchers reported greatly enhanced MSC trophic activities upon culture with cellulose-fiber-concentrated CM[139]; pilot experiments testing enhancement of growth factor concentrations revealed that relevant growth factor concentrations did not scale with volume depletion. Further, trophic effects of concentrated-CM were not markedly better than non-concentrated CM. For this reason, CM, without further manipulation, was utilized for all experiments.

To promote better DRG attachment and growth on NFC substrate, laminin- and lysine-coatings were utilized, in line with a growing consensus that many aligned fibrous structures bearing ECM molecules are beneficial to the ultimate function of a nerve guide conduit[207]; several groups have also investigated physical modifications to the PCL surfaces or

chemical/ligand conjugation [76,208] to better support initial nerve attachment and proliferation, and work is ongoing with coatings derived from various extracellular matrix (ECM) molecules. While fiber culture was ultimately found to be detrimental to MPC-neurotrophic induction but not survival (Figure 13), construct-seeded MPCs preferentially supported DRG-neurite extension, and the combination of GM-EC and NM-MPC co-culture enhanced this support (Figure 14). This suggests that by optimizing the interactions of MPCs and ECs, preferential nerve support can be achieved. Proteomic analysis of the secretomes of both cell types might elucidate synergistic neurotrophic interactions or potential antagonistic interactions between inhibitory factors in either the MPC- or EC-CM, the details of which warrant further study.

In addition to its well established activity as an angiogenic factor, VEGF has been known to affect the neural system[33,209]. We therefore examined the potential involvement of VEGF in the neurotrophic support of MPC-CM and EC-CM. Following immunoprecipitation of VEGF from MPC-CM and EC-CM, neurite extension decreased when compared to the use of control antibodies, suggesting that VEGF is required for the promotion of neurite outgrowth (Figure 16). IgG control-treated CM, regardless of source, elicited longer neurite extensions. Analysis of the control antibody formulation revealed the presence of gelatin versus bovine serum albumin in the control antibody formulation versus the anti-VEGF antibody formulation. These protein differences could presumably affect the net neurotrophic activity, and further investigations of the composition of the CM after VEGF removal will therefore need to be performed. Additionally, both neurite VEGF sensitivity (as measured by the density of VEGFR surface expression) and available VEGF (as indicated by the absence of soluble and membrane-expressed VEGFR-1) may be affected by the selective removal of human VEGF. Studies

examining those aspects of neurite VEGF responsiveness might shed more light on the exact mechanism of MPC-produced VEGF neurotrophic support.

Within the cell suspension isolated from traumatized muscles, a CD29+/CD34+ vascular endothelial progenitor cell (EPC)-like population has been noted[143]; muscle-derived stem cells have additionally been observed to spontaneously express both vascular and neurotrophic markers upon culture with neurons[210], suggesting plasticity or a mixed culture of these cell types[207,210]. The preferential support by MPCs of nerve cells suggests that the above work could be explained by the presence of MPC-derived EPCs.

Taken together, these findings demonstrate that the neurotrophic activities of MPCs can be enhanced by chemical induction, CM-combination with ECs, or co-culture with adult ECs. VEGF played a role in the observed positive effect of MPC-CM on DRG neurite extension; given its dual role as an angiogenic and neurotrophic factor coupled with the observation of enhanced neurite extension under EC influence, future NFC scaffolds could incorporate VEGF in an attempt to support both cell types. The detrimental effect of the fibrous scaffold on MPC gene expression and measured protein secretion indicates that PCL, in particular, is a sub-optimal substrate for NM-MPCs. Work with more cell-friendly substrates, including thin ECM-coatings on the existing PCL scaffold structure, is both ongoing and shows promise in the development of a regenerative medicine construct for nerve repair.

4.0 INFLUENCE OF INFLAMMATION AND ENDOTHELIAL CELLS ON *IN VITRO* OSTEOGENESIS OF TRAUMATIZED MUSCLE PROGENITOR CELLS

4.1 INTRODUCTION

Within the first 48 hours after traumatic injury, increases in circulating levels of pro-inflammatory molecules, including interleukin (IL)-6, IL-1β, tissue necrosis factor (TNF)-α, macrophage-interaction promoting IL-18, C-reactive protein (CRP), sepsis-correlated procalcitonin, and anti-inflammatory IL-10, are observed[211]. Previous studies have shown that excess amounts of IL-6 have a detrimental effect on the hallmark-ability of adult tissue-resident stem cells to differentiate along mesenchymal (adipose, cartilage, bone) lineages; IL-6 promotes "stemness" and cell replication over differentiation[172] and is produced endogenously by microvascular endothelial cells (ECs)[212]. IL-1β and TNF-α show similarly detrimental effects on mesenchymal differentiation with slightly decreased proliferation of mouse bone marrow-derived mesenchymal stem cells (MSCs)[213].

We have previously identified multipotent progenitor cells (MPCs) in blast-traumatized human skeletal muscle.[163] These cells are characterized by their ability to differentiate along hallmark mesenchymal lines and their similar surface epitope profile compared to human bone marrow MSCs[163]. Although more easily-isolated and more abundant per unit tissue[134] than bone marrow- or adipose-derived MSC subtypes, suitable MPC-donor tissue may be limited due to the

severity of some limb-blast injuries; this potential limitation requires extensive *in vitro* expansion of any isolated MPC to obtain sufficient cell number for use in a regenerative medicine therapy. Culture-related changes in MSC stem cell gene expression and DNA methylation but not stem cell surface markers have been reported[214,215]. Previous work indicated no stem cell marker population differences between MPC and MSC at low passage number[139] as revealed by flow cytometry; however, slight differences in fluorescence intensity of each marker were observed[139]. It has been noted that MSCs do not lose stem cell marker expression upon long-term cultures, even if their differentiation capacity has been compromised[214].

Interestingly, the inflammatory factors IL-1β and TNF-α are upregulated in MPCs versus non-traumatized muscle cell controls[213]. In bone marrow-derived MSCs, a possibly related and more extensively characterized stem cell population that also exhibits bone-forming activity, low levels of TNF-α (<10 ng/ml) appear to enhance early osteogenesis, indicated by alkaline phosphatase (ALP) expression and early osteogenic gene expression, while high doses (20-50 ng/ml) of TNF-α suppress osteogenesis and encourage a neural phenotype through nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling. In comparison, IL-1β appears to suppress osteogenic gene expression but promote mineralization (usually a marker of mature osteogenesis) at low doses, with unknown activity at high doses[216]. One of the well-recognized tissue responses to traumatic injury is heterotopic ossification (HO), observed most commonly in skeletal muscle sites adjacent to the traumatized site.[217] While the exact etiology of HO is incompletely understood and is currently being investigated[218], it is likely that the presence of inflammatory factors enhances the osteogenic differentiation of the resident MPCs in skeletal muscles.

Another possible influence on MPC osteogenesis may be from the vasculature, i.e., endothelial cells. Endothelial cellshave been observed to affect the osteogenic differentiation of MSCs; co-cultures of MSCs with endothelial cells showed accelerated bone phenotypes[216,219]. Endothelial cell activity, however, is known to be affected by inflammation; specifically, IL-1β and TNF-α disrupt stable endothelial networks (*in vitro* markers of angiogenesis) noticeably (5 ng/ml TNF-α) or slightly (5 ng/ml IL-1β), and alter the proliferation of endothelial cells[173]. The effect of these disrupted angiogenic systems on MPC osteogenesis is unknown.

In turn, MSCs and MPCs are known to regulate angiogenesis. MPCs cause proliferation of ECs[141], and various types of MSCs have noted anti-inflammatory properties and can stabilize endothelial cell networks[220,221]. Additional, new observations on the effect of MPCs on the organization of endothelial networks and other aspects of angiogenesis are reported in the second chapter.

The studies described above clearly suggest that endothelial cells and MSCs interact dynamically and regulate one another in the wound environment. It is thus reasonable to postulate that this paracrine regulation, in turn, affects many aspects of tissue healing and regeneration including, but not limited to, osteogenesis and angiogenesis; these interactions have not been characterized with respect to MPCs. As MPCs stabilize the angiogenic functions of endothelial cells, it is hypothesized that endothelial cells reciprocally stabilize the osteogenic differentiation capacities of MPCs, dampening the deleterious effects of an inflammatory environment and promoting a mature, osteogenic MPC-phenotype. This hypothesis is tested here by examining the effect of adult human dermal EC-modulated inflammation on the osteogenic differentiation of MPCs and MSCs. While inflammation promoted a unique, mineralizing, but

immature phenotype, the presence of ECs or EC-conditioned medium (EC-CM) aided the maturation of MPC cultures or co-cultures.

4.2 METHODS

4.2.1 Primary MPC isolation

MPC2-4 (Appendix A) were obtained following debridement of blast-traumatized wounded U.S. military personnel with Institutional Review Board (IRB) approval (Walter Reed National Military Medical Center). Fascia-free minced muscle tissue (200 μg) was enzymatically digested for 2 hours (Dulbecco's Modified Eagle's Medium, DMEM; GIBCO; 0.5 mg/ml collagenase 2, Sigma) at 37°C under gentle agitation, as originally described by Jackson, et al[163]. MPC were strained through a 40 μm cell strainer and pelleted by centrifugation at 200 x g for 5 minutes. Cells were resuspended in DMEM, 10% stem cell culture-approved fetal bovine serum (FBS; GIBCO), and 5% penicillin-streptomycin (PSF; GIBCO), plated in a T150 tissue culture flask (Corning), and allowed to adhere overnight at 37°C and 5% CO_2. Cells were then washed extensively with phosphate-buffered saline (PBS; GIBCO) and cultured in DMEM, 10%FBS, and 3% PSF, with daily PBS washes, at 37°C under 5% CO_2 until confluent.

4.2.2 Primary MSC isolation and storage

MSC3-5 (Appendix A) were obtained after flushing scissor-minced trabecular bone removed from the femoral heads of patients undergoing total hip arthroplasty (IRB approval, University of

Washington) rinsing medium (α-Minimal Essential Medium: α-MEM, 1% PSF; GIBCO). The cell-enriched rinsing medium was strained through a 40 μm cell strainer, and the resulting cells pelleted at 300 xg for 5 minutes. Cells were washed with rinsing medium and pelleted twice before resuspension in growth medium (rinsing medium + 10% FBS + 1 ng/ml basic fibroblast growth factor: FGF-2; Fisher). Cells were allowed to adhere to T150 tissue culture flasks and cultured in growth medium at 37°C under 5% CO_2 until confluent.

Upon reaching confluency, cells were washed in PBS, trypsinized (Invitrogen), counted by a calibrated, automated cell counter via Trypan Blue exclusion (CedEx; Roche), and frozen (-200°C) at $1x10^6$ live cells/vial in 1 ml Freezing medium (Invitrogen) at passage 0 (p.0). Passage numbers were incremented upon thawing.

4.2.3 Cell culture expansion

All expansions were performed on tissue culture-treated plastic T150 or triple flasks (Nunc/BD) at 37°C in 5% CO_2. MPCs and MSCs were maintained in growth medium, exchanged every 3-4 days, for expansion. ECs were expanded using EGM-2MV Bullet Kit (Lonza). Upon reaching 70-90% confluency, cells were trypsinized, counted, and seeded into new flasks, utilized for experiments, or frozen at 1E6/vial (Freezing medium: Invitrogen).

4.2.4 CM generation

ECs were pre-seeded at an initial density of $1x10^4$ cells/sq. cm in EGM2-MV for 24 hours. Just prior to incubation with IL-1β-supplemented or non-supplemented basal medium

(DMEM+1%PSF+1% Insulin-Selenium-Transferrin-X; Invitrogen), cells were washed with PBS and Hanks-buffered salt solution containing calcium and magnesium (HBSS++; GIBCO). EC-conditioned medium (EC-CM) with supplements of 5- or 10 ng/ml IL-1β were generated by incubating ECs with serum-free basal medium for 48 hours. Non-cell conditioned control CM was generated by incubating basal medium with or without IL-1β in empty flasks at 37°C under 5% CO_2. All CM samples were centrifuged at 200xg for 5 minutes before the supernatant was frozen at ≤-20°C for later use. At the time of use, CM samples were thawed, centrifuged at 200 xg for 5 minutes, and filtered through 0.2 μm filters (Steri-flip; Millipore) before use.

4.2.5 Inflammation/angiogenesis-moderated *in vitro* osteogenesis

Conditioned medium (CM) generated with ± 5 ng/ml IL-1β (± I) was used to supplement the medium of MPC/MSC or EC-co-cultures undergoing *in vitro* osteogenesis using a standard cocktail (Final composition: DMEM+10% FBS+2% PSF+50 μg/ml ascorbic acid, 1 nM dexamethasone, 10 mM betaglycerophoshate; all supplements from Sigma-Aldrich) as described below (Figure 17). Cells were seeded at $2x10^4$ cells/cm^2 in tissue culture-treated 24- or 48-well plates (Costar). Medium was changed every 2-3 days.

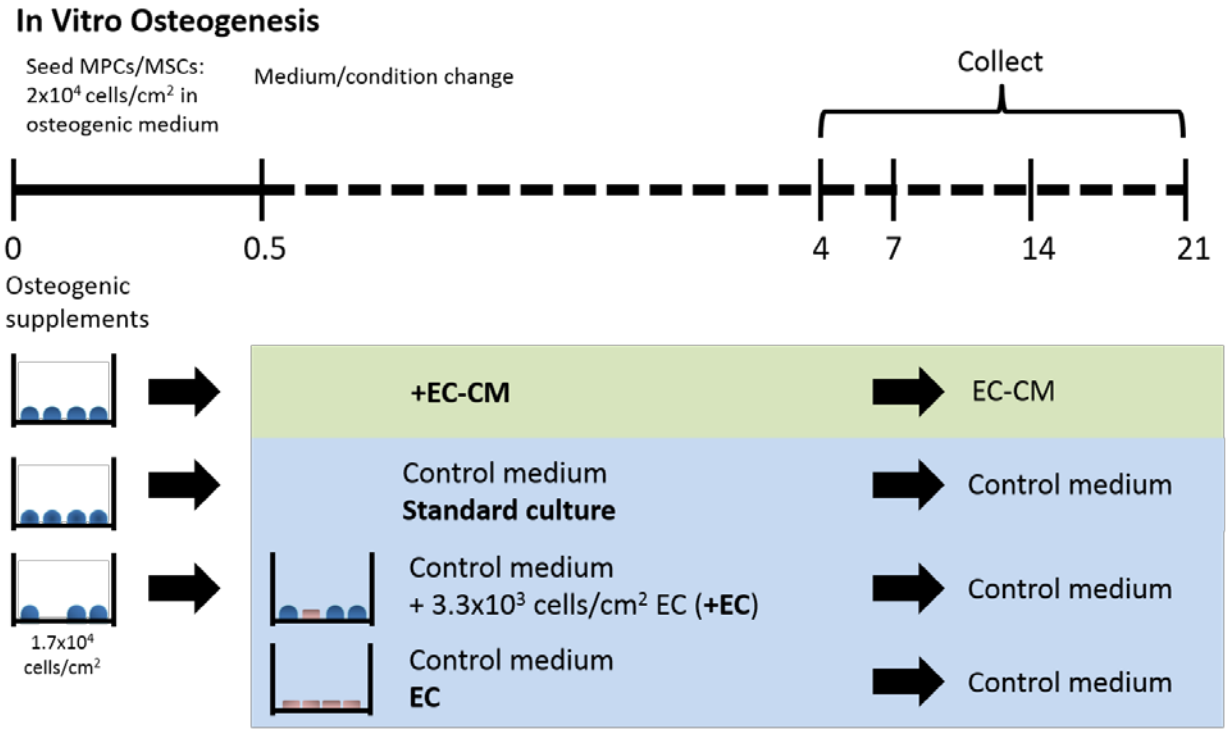

Figure 17: Effect of conditioned medium on MPC and MSC osteogenic differentiation
Unless noted, all cells were cultured for up to 21 days under osteogenic conditions by adding osteogenic supplements to CM generated under cell-free conditions (blue control medium). MPCs or MSCs, represented as dark blue cells, were seeded at $2x10^4$ cells/cm^2; ECs were seeded in control cultures at the same density and are represented as red cells. For co-cultures, MPCs or MSCs were loaded at 5/6 of the original seeding density, and the remaining 1/6 of the culture filled by ECs after the firm attachment of MPC/MSC (denoted by +EC). The effect of EC-CM and EC-CM+I was also tested on MPC-only and MSC-only cultures (green bar).

4.2.6 Osteogenesis assessment

Cell lysate obtained using 0.25 ml 0.4% SDS in diH2O per well of a 48-well plate was used to quantify alkaline phosphatase activity (p-nitrophenyl phosphate substrate; NPP, Sigma), which was normalized to DNA, extracted using the Allprep protocol (Qiagen) and measured using the Pico Green assay (Invitrogen). Reported cellularity was normalized to culture surface area and equivalent ALP cell lysate concentrations; co-culture specific activity was further normalized to the initial amount of osteogenic cells (MPC or MSC).

Osteogenesis in cultures was assessed after paraformaldehyde fixation at early time points (day 7) with alkaline phosphatase staining (Sigma kit). Paraformaldehyde-fixed samples were stained with Alizarin Red S (EMS) to visualize late osteogenesis calcium deposition at day 21. All stained samples were visualized on an Olympus CKX41 inverted microscope at 200x. Relative amounts of matrix-bound Alizarin Red S were quantified by incubating thoroughly washed samples with 10% acetic acid for 30 minutes and measuring absorbance (550 nm) of centrifuged (200 xg), isopropanol-diluted supernatant.

4.2.7 Gene expression analysis

RNA was extracted from samples using RLT buffer supplemented with 10 nM β-mercapto-ethanol (Qiagen; Bio-Rad). RLT-lysed cells were stored at -80C. Upon thawing, samples were processed with RNEasy (or Enzymax brand) spin columns, according to the manufacturer's instructions. RNA purity and concentration were determined by 260/280 and 260/230 readings

(NanoDrop 2000C). RNA was converted to cDNA using SuperscriptIII with random hexamers (Invitrogen; BioRad iCycler) and stored in small, pooled aliquots at -80C. cDNA corresponding to 10 ng original RNA was loaded in triplicate wells of a 96 well qPCR reaction plate with 1x SYBR Green Master Mix (Invitrogen) and 100 nM each forward- and reverse- primers (Table 2); following 40 thermal cycles between 95-60C (ABI 7900 HT; ABI StepOne Plus), software-computed osteogenic or EC-specific expression threshold values were compared to a pooled cDNA standard curve (used to normalize expression between plates) to determine gene expression compared to 18S control genes. Ct values higher than 35 were rejected.

Table 2: Primer sequences for qPCR analysis

Gene	Primer sequence (5' – 3')*	
	Forward	Reverse
18S ribosomal RNA (18S)	-GTAAC CCGTT GAACC CCATT-	-CCATC CAATC GGTAG TAGCG-
Alkaline phosphatase (ALP)	-TGGAG CTTCA GAAGC TCAAC ACCA-	-ATCTC GTTGT CTGAG TACCA GTCC-
Bone morphogenetic protein 2 (BMP)	-ACCCG CTGTC TTCTA GCGT-	-TTTCA GGCCG AACAT GCTGA G-
Bone sialoprotein II (BSP-II)	-GCAGT AGTGA CTCAT CCGAA GAA-	-GCCTC AGAGT CTTCA TCTTC ATTC-
Collagen, Type I, α1 (COL)	-TCCTG CTCCT CTTAG CG-	-CATGGT ACCTG AGGCC GTTC-
Osteocalcin (OCN)	-TCACA CTCCT CGCCC TATTG-	-GAAGA GGAAA GAAGG GTGCC-
Runt-related transcription factor 2 (RUNX2)	-CAACC ACAGA ACCAC AAGTG CG-	-TGTTT GATGC CATAG TCCCT CC-
Cluster of differentiation 31 (CD31)	Proprietary sequences**	

* Purchased from IDT. **RT^2 Profiler primers purchased from Qiagen.

4.2.8 Stem cell marker surface expression over time in culture

At low (3) and high (10) passage, MPC and MSC (n=3) were trypsinized, inactivated with FBS, and resuspended with FACS buffer (Ca^{2+}- and Mg^{2+}-HBSS buffered salt solution + 5 ml FBS + 0.01% sodium azide: Invitrogen). After blocking with 10% mouse serum in HBSS, $2.5x10^5$ FACS buffer-washed MPC were incubated with appropriate antibodies (Table 3) and fixed in 4% paraformaldehyde. Marker expression was analyzed on a BD FACSAriaII, with cell- and passage-dependent gating determined by an experienced, independent analyst based on IgG-matched patient controls.

Table 3: Flow cytometry markers and staining conditions*

PE-conjugated antibody target	BD Biosciences catalog number	Antibody distribution (µl/1M cells)	Literature-reported MSC threshold[115]
CD19	555413	20	<5%
CD34	550619	5	<5%
CD45	557059	20	<5%
CD73	550257	20	>95%
CD90	555596	0.2	>95%
CD105	560839	20	>95%
HLA-DR	560943	20	<5%
IgG-1a isotype control	554680	assay-dependent	-
CD14	555574	20	<5%
IgG2-a isotype control	558595	assay-dependent	-

* Negative markers exclude MSCs from hematopoietic, monocyte/macrophage, B-cell, or leukocyte origin, and in the case of HLA-DR, exclude inflammation-activated ('stimulated') MSCs[121]. Positive markers are not unique to MSCs, but associate MSCs with the vasculature, fibroblasts or activated endothelium, and mineralizing ability[220–222].

4.2.9 Statistical analysis

Data were analyzed in Excel and GraphPad Prism. Data were presented as mean ± standard deviation. Outliers were removed with Grubbs' test. Student's t-tests were used for comparison when 3 or fewer conditions were compared. For larger data sets, GraphPad Prism was used to perform one- or two-way ANOVA with multiple comparisons, using Sidak's or Tukey's corrections for multiple comparisons, respectively.

4.3 RESULTS

4.3.1 Generation of inflamed endothelial cells

To test whether IL-1β-treated ECs were affected by the presence of the cytokine, ECs plated on Matrigel were treated with 5 and 10 ng/ml IL-1β. As shown in Figure 18, IL-1β treatment was seen to result in shortened and disrupted EC networks. Because the lower concentration was functionally effective, 5 ng/ml was chosen for subsequent experiments involving inflamed ECs. The IL-1β effect was also confirmed by comparing angiogenic gene expression via PCRArray of EC grown in basal medium (CM) to EC grown in basal medium with IL-1β supplementation (CM+I) for 2 days. Despite equal initial seeding density, EC+I cultures contained fewer cells than control cultures by the end of the incubation period used for CM collection, consistent with literature observations[173]. Specifically, three genes were significantly (>2-fold, $p<0.05$) upregulated in IL-1β cultures over control cultures: chemokine C-X-C ligand (CXCL)-6,

interferon (IFN)-β1, and insulin-like growth factor (IGF)-1. IL-1β was not detected in EC-CM, and the concentration of IL-1β had dropped to 59 ± 12 pg/ml in EC-CM+I, which was still well within the IL-1β concentration range reported to affect MSC *in vitro* osteogenesis[97].

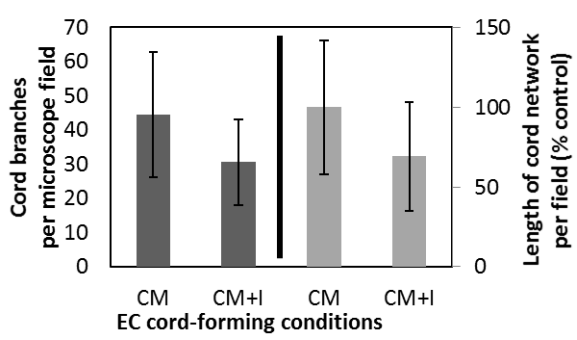

Figure 18: Effect of IL-1β on EC cord formation on Matrigel
Treatment with 5 ng/ml IL-1β was sufficient to decrease EC Matrigel cord-formation estimated based on cord branching and length of cord network.

4.3.2 Inflammation associated osteogenesis: Osteogenic gene expression

To assess the effect of IL-1β treatment on osteogenesis, osteogenic gene expression was assessed after 4 and 14 days in osteogenic cultures of MPCs and MSCs supplemented with cell-free CM or CM+I (Figure 19). When normalized to day 4 growth osteogenic marker expression, all osteogenic markers increased with time in culture. Early osteogenesis markers, ALP and RUNX2, were expressed more strongly by MSCs than MPCs. Expression in both cell types was decreased due to inflammation, consistent with previous reports[225]. MPCs did not express BSP-II at detectable levels. Interestingly, MPCs exhibited higher levels of OCN, COL-I, and BMP2 gene expression than MSCs. This indicates that MPCs might encourage bone formation rather than creating bone themselves.

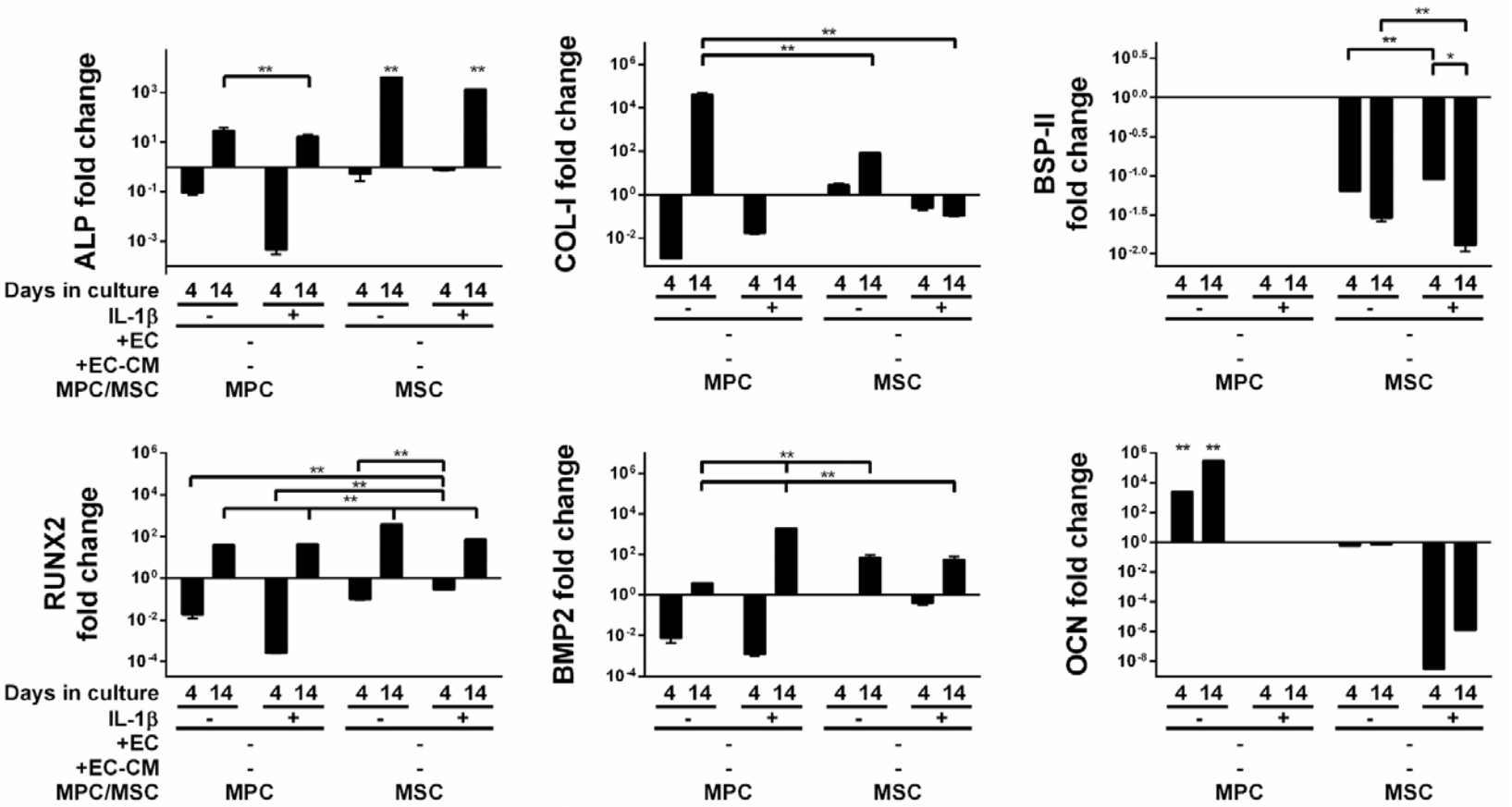

Figure 19: Inflammation decreased osteogenic gene expression in MPC and MSC cultures

Osteogenesis of MPCs and MSCs was assessed on the basis of RT-PCR analysis of gene expression after 4 and 14 days in culture. For cultures with and without IL-1β, pooled MPC- and MSC- ALP, RUNX2, collagen-IA1 COL-I, BMP2, BSP-II, and OCN gene expression were examined at d.4 and d.14; marker expression was normalized to 18S and d.4 growth gene expression; n=3, **p<0.01, log scale. Osteogenic gene expression increased with culture time, and the presence of IL-1β contributed to lower osteogenic gene expression when compared to non-inflamed controls at the same timepoint. Mature osteogenic gene expression was less reliable in MPCs when compared to MSC, frequently falling below the assay detection limit (Ct > 35). CD31 gene expression was initially high in EC cultures and low in MPC/MSC cultures but dropped below the assay detection limit by day 14.

4.3.3 EC co-culture and survival

To more directly address the bone-forming abilities of MPCs in a physiological context, osteogenic cells were next seeded in co-culture with ECs. To address the relative contribution of seeded cells to observed culture activity, it was necessary to determine the relative amount of each cell in co-culture. The survival of CD31+ ECs was estimated by ELISA of cell lysates prepared in the presence of protease inhibitors (Figure 20).

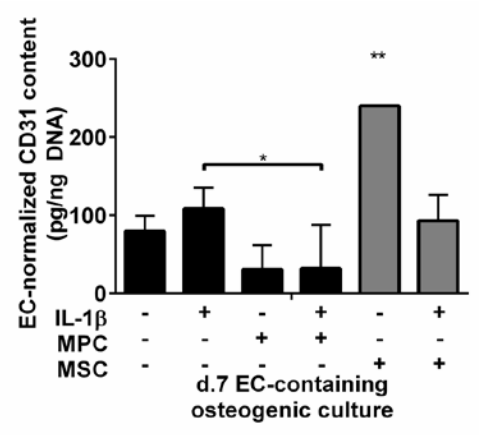

Figure 20: Persistence of ECs in monoculture and co-culture with MPCs/MSCs upon treatment with IL-1β
CD31 was detected in EC monoculture and co-culture cell lysate and normalized to the initial EC population. Decreased specific CD31 production (production/cell) was observed in all co-culture groups with increasing culture time, and specific CD31 content increased slightly due to IL-1β presence except in MSC co-cultures.

CD31 cell content increased in EC cultures but decreased in co-cultures over time. The decline was increased by IL-1β exposure. This indicates a loss of EC or EC-CD31 production over time, either through MPC/MSC preferential proliferation, EC CD31-downregulation, or

MPC-EC/MSC-EC cell fusion[226]. Evidence from CD31 immunofluorescence suggests the latter two possibilities, as global CD31+ staining was observed in co-cultures after 24 hours. While EC cell death could also explain the CD31 loss, the magnitude of CD31 loss does not correlate with the culture cellularity (Figure 21). Per-cell DNA content did not vary significantly by cell type at the start of osteogenic culture; however, MSCs proliferated rapidly, reaching ~4x the cellularity of MPCs by day 4 and maintaining that difference throughout the culture period.

4.3.4 Functional effects of inflammation and co-culture: ALP activity

Because ALP activity, along with exogenous phosphate, is required for *in vitro* osteogenesis[94], it was assessed at an intermediate time point (day 7) in EC-influenced co-cultures. Consistent with observations reported elsewhere[98], decreases in ALP production due to IL-1β could be observed by Naphthol AS-BI ALP-staining and quantified by osteogenic cell-normalized NPP conversion (Figure 22 A and B). Surprisingly, EC co-culture further diminished observed ALP activity, but these decreases could be partially rescued by the addition of EC-CM. ALP activity could be attributed almost solely to MPCs or MSCs because EC-specific ALP activity was negligible. This suggests that MPCs are uniquely responsive to soluble factors released by endothelial cells, and that such factors could promote bone formation by MPCs.

4.3.5 Functional effects of inflammation and co-culture: Mineralization and selected gene expression

To assess the mineralization of osteogenic co-cultures, cultures were stained at day 21 with calcium-binding Alizarin Red S. After imaging, mineralization was quantified by solubilizing the Alizarin Red S (Figure 23). MPC+EC+I exhibited the strongest mineralizing phenotype among MPC cultures. Gene expression of ALP, RUNX2, OCN, and BMP2 in MPC+EC+I was highest among all 14-day cultures groups (Figure 24), indicating that MPCs in an inflamed and angiogenic environment may contribute preferentially to de novo bone formation. EC-CM affected osteogenic cultures similarly but to a lesser extent than EC co-cultures (Figure 25).

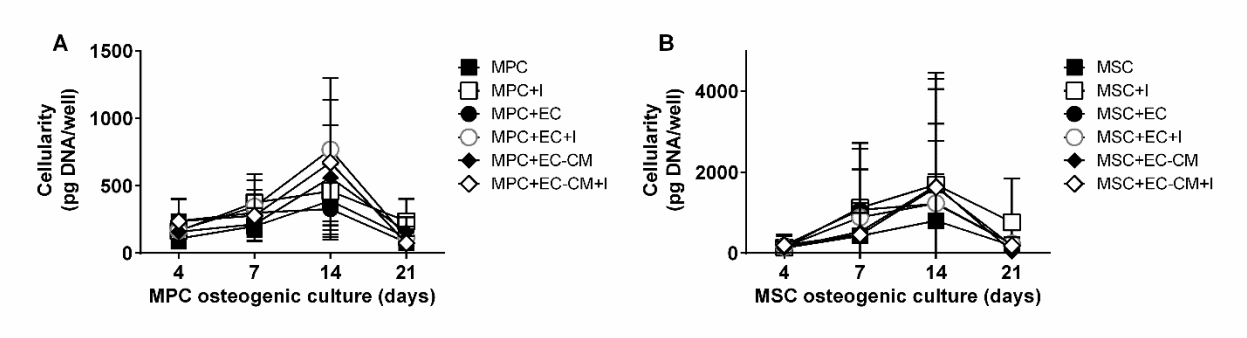

Figure 21: Osteogenic culture cellularity of (A) MPCs and (B) MSCs during osteogenic differentiation *in vitro*
Cellularity based on DNA analysis did not vary significantly between culture groups at each measured time point. All cell types exhibited similar DNA content per cell on day 0. MSC proliferated faster than MPC, as cellularity measurements at nearly all timepoints were roughly four times larger in MSC- versus MPC-cultures (including growth controls). The observed decrease in isolated DNA at day 21 could be a result of either cell death or double-stranded DNA-depletion in solution, based on binding to insoluble products or DNA destruction due to increased shear. Day 21 samples required much more force to break apart the dense mineral and cell aggregates that had formed.

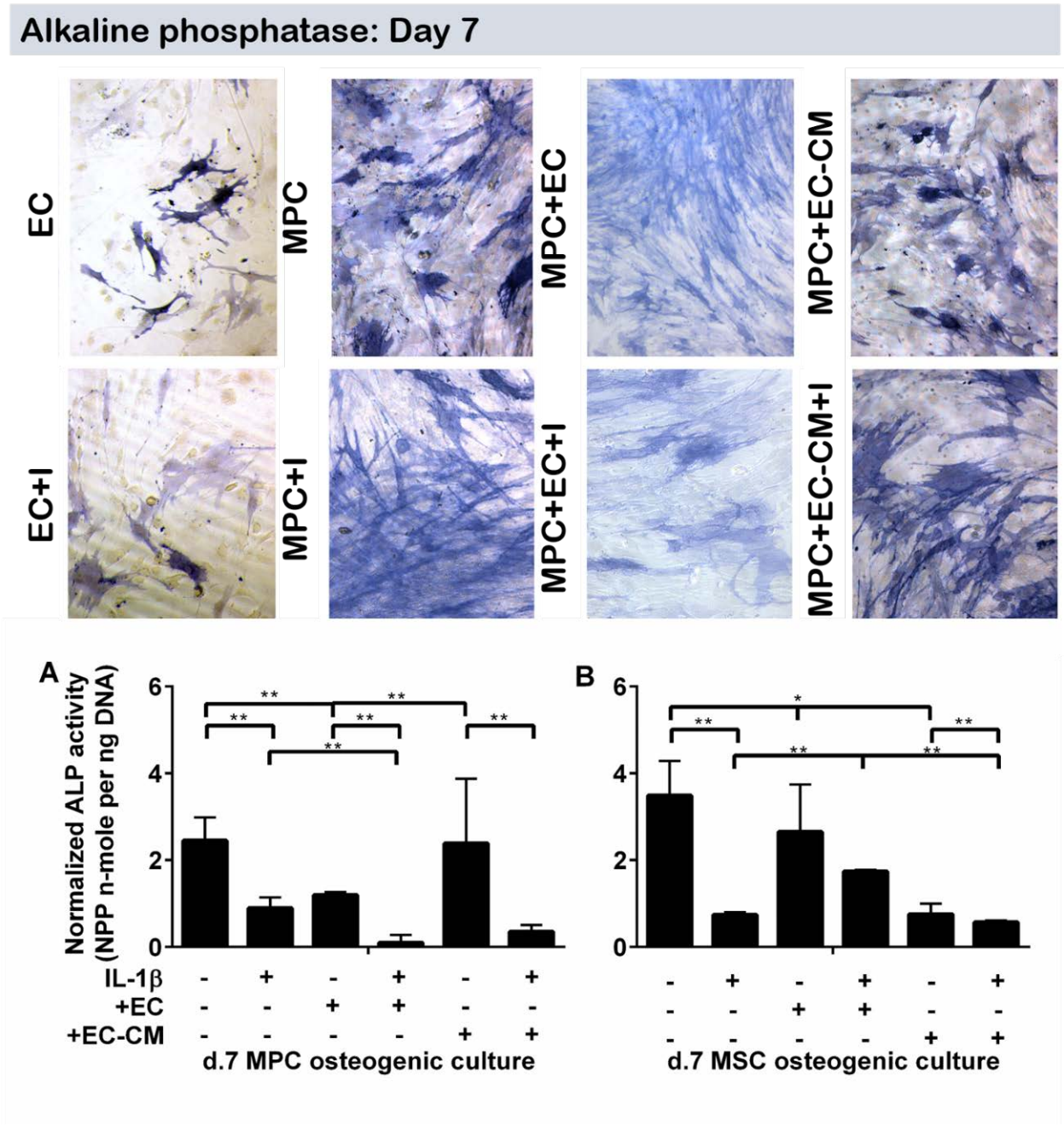

Figure 22: Inflammation decreased ALP activity in MPC/MSC cultures regardless of EC co-culture or EC-CM

(Top) Early time-point osteogenic cell-normalized Naphthol AS-BI staining (Top) and ALP activity (Bottom; as indicated by NPP conversion). n=3; *,$p<0.05$; **, $p<0.01$. Because EC-ALP activity was extremely low, co-culture specific ALP was normalized to the initial amount of osteogenic cells. The presence of IL-1β inhibited ALP activity, and the effect could not be overcome by EC influence (either co-culture or EC-CM). EC-CM partially rescued MPC ALP activity, but not MSC, suggesting that MPC are more easily routed towards an osteogenic fate by EC-produced soluble factors.

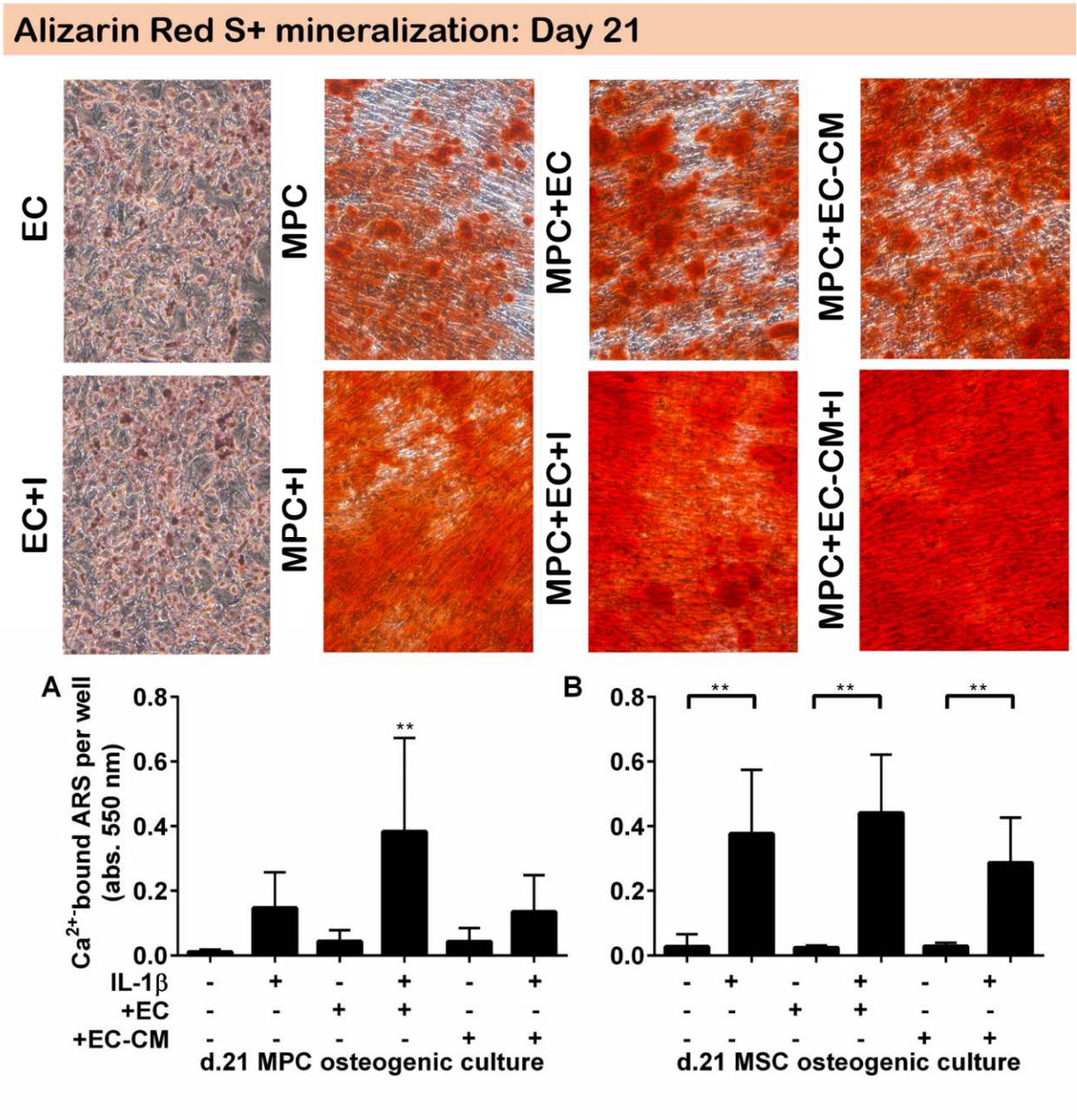

Figure 23: Inflammation overwhelmed the influence of EC co-culture and EC-CM on MPC/MSC osteogenesis *in vitro* as indicated by matrix mineralization

(Top) Alizarin Red S (ARS) staining, and (Bottom) quantification of (A) MPC and (B) MSC mineralization revealed a strong IL-1β-dependence with a much stronger mineralization response by MSCs versus MPCs; n=3, **, $p<0.01$. EC co-culture and EC-CM appeared to have less of an effect on mature osteogenesis when compared to IL-1β, and the combination of MPCs and ECs resulted in a strongly mineralizing phenotype. Note that comparable mineralization was achieved by ~1/4 the number of MPCs versus MSCs in the MPC+EC+I culture.

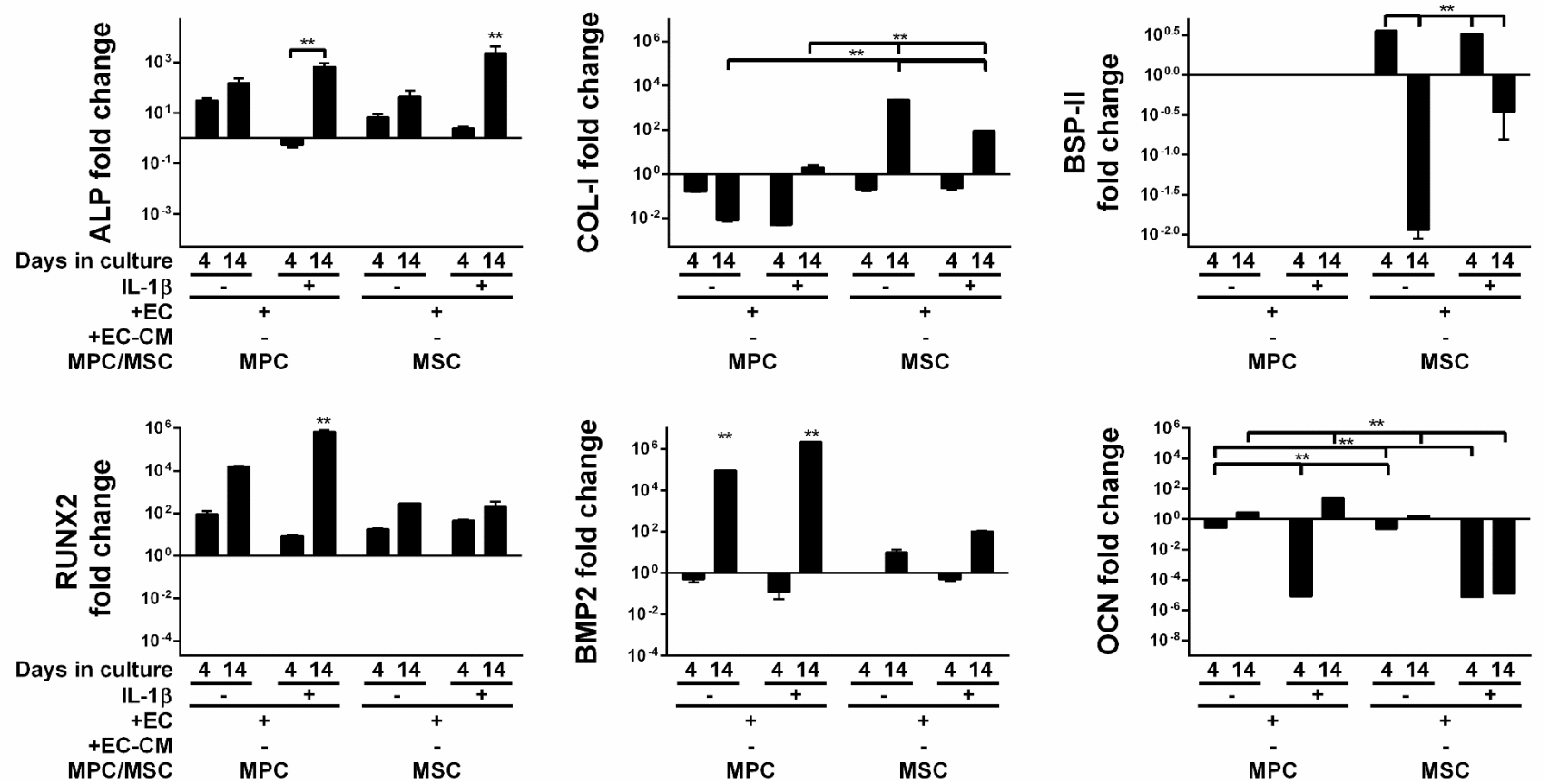

Figure 24: Osteogenic gene expression increased due to inflammation in MPC- and MSC- EC co-cultures
Some of the strongest osteogenic gene responses were observed in MPC-EC co-cultures at d.14. Except for MSC late osteogenesis markers, IL-1β increased osteogenic expression in co-cultures by d.14, indicating that inflamed-ECs interact with MPCs to promote osteogenesis, but EC effect on MSC is less strong.

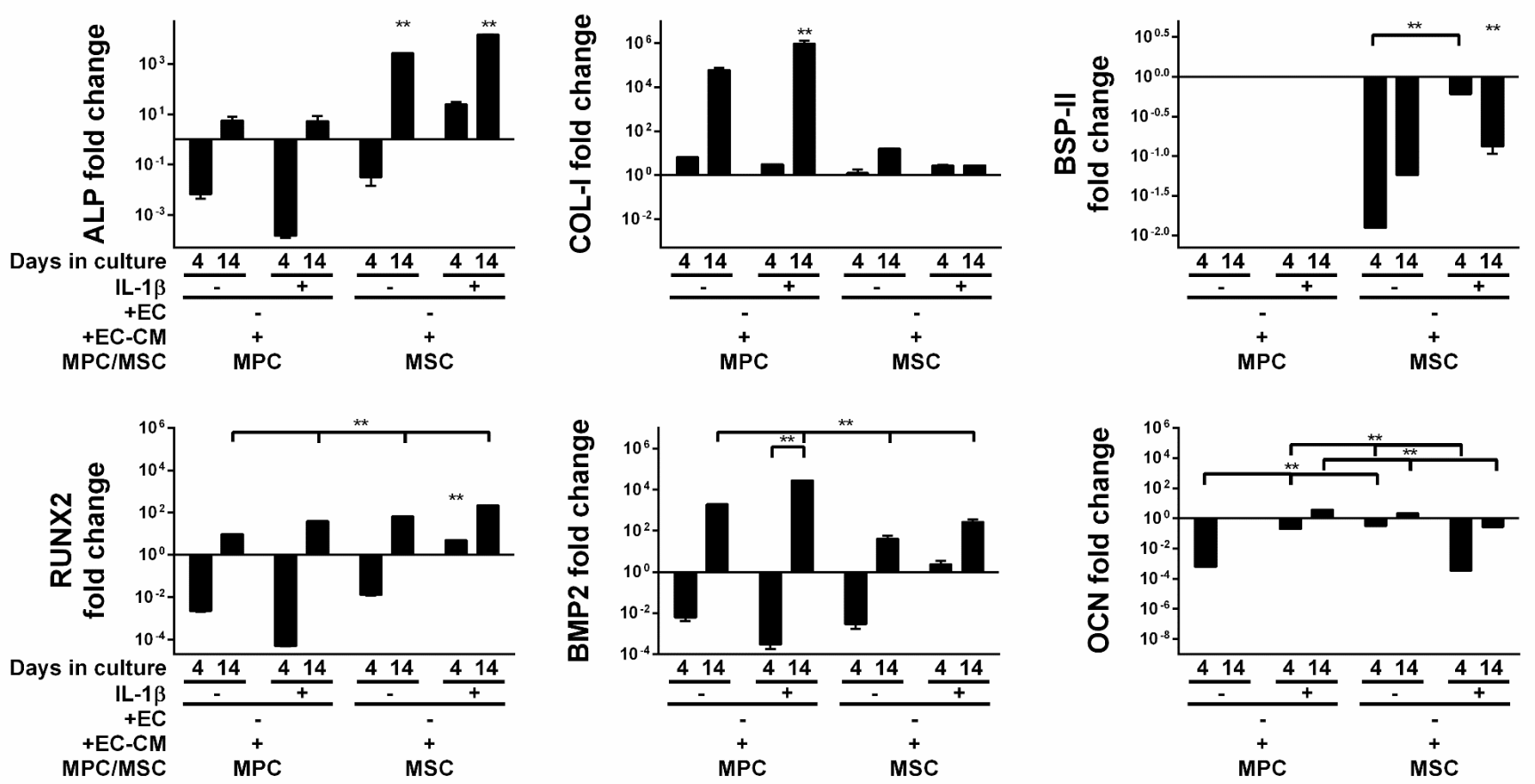

Figure 25: Osteogenic gene expression increased due to inflammation in EC-CM-treated MPC and MSC cultures
Gene expression patterns were similar to those observed without EC-CM with respect to IL-1β effects; EC-CM appeared to slightly decrease early osteogenic genes (ALP and RUNX2) and induce stronger BMP2, BSP-II, and OCN gene expression by day 14 when compared to non-EC-CM cultures. This indicates that EC-CM may drive MPCs and MSCs towards a more mature, mineralizing phenotype.

4.3.6 Stem cell marker expression over time

Because differentiation ability can be lost with *in vitro* age[214], MPC and MSC stem cell marker expression was assessed at early and late cell passage (p.3 to p.10). One patient from each studied group was excluded due to extreme loss of stem cells markers by p.10 (Figure 26). Although statistical comparisons were not possible, trends indicating enrichment of $CD34^+$ MPCs and maintenance of or increases in stem cell marker expression were observed. This indicates that tissue-resident or tissue-recruited progenitor cells may not strictly be termed MSCs despite their differentiation and trophic abilities; if possible, cells from more patients should be investigated to draw conclusions about MPC versus MSC differences in stem cell marker expression.

Analysis of work from other chapters revealed that the excluded populations, MPC1 and MSC3, were often excluded via outlier testing from contributing to global descriptions of MPC or MSC activities. This affirms the need for careful functional testing, including the effect of extended proliferation, of autologous cells before they are utilized therapeutically.

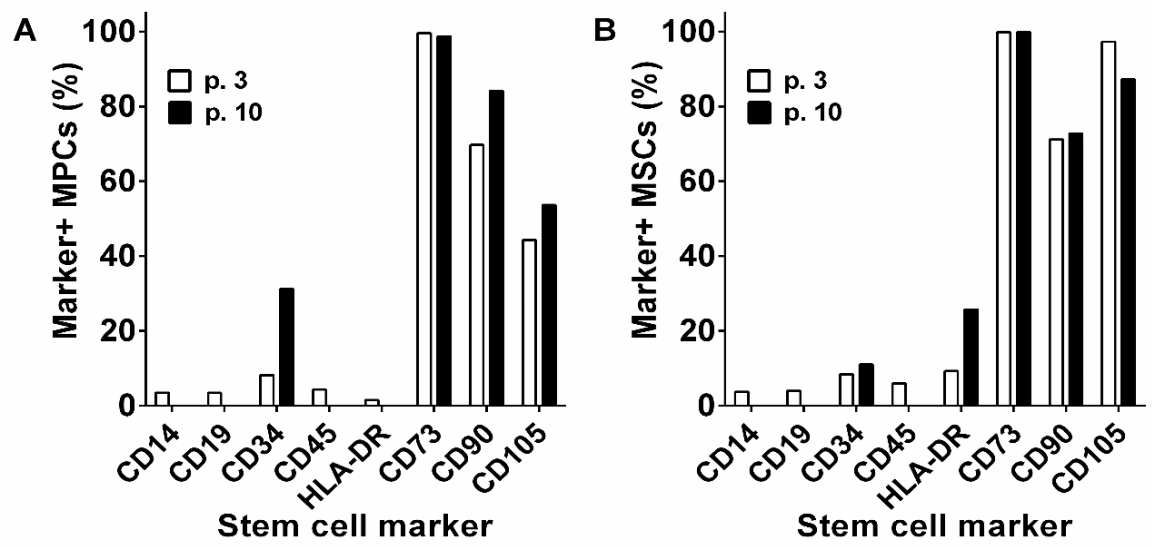

Figure 26: Stem cell marker profile changes as a function of MPC and MSC culture time
Single replicate changes in flow cytometry measurements of stem cell markers were assessed in MPC (A, C) and MSC (B, D). Neither studied population strictly fit the minimum definition of a stem cell despite functional MSC-screening. Except for CD34 which increased with cell passage in both populations, MPCs stem cell marker expression appeared to drift towards full stem cell marker expression (loss of CD14, CD19, CD45, and HLA-DR with gains in CD90 and CD105 expression) with increasing passage; MSC stem cell marker expression also drifted towards increased stem cell marker expression, except for gains in HLA-DR expression coupled with a loss of CD105.

4.4 DISCUSSION

The use of autologous multipotent cells for limb repair is an intuitive and desirable therapeutic goal. There is mounting evidence that the highly-inflamed and angiogenic environment of a blast-traumatized wound bed serves as a potent source of culture-expandable, uniquely active traumatized multipotent progenitor cells, the MPCs. Our recent and past work has explored the effect of *in vitro* conditioning of these cells for eventual therapeutic purposes, with some results hinting at their possible involvement in blast-associated diseases[136]; in other words, there is concern over the biological activity states of these cells, once they are returned to a wounded tissue microenvironment. The current work describes *in vitro* studies aimed at elucidating changes to MPC osteogenic activities induced by their environment, chiefly in the context of both inflammation and angiogenic influences. In addition, MPC activities were compared to better-characterized mesenchymal stem cells (MSCs), such as bone marrow-derived MSCs in an attempt to identify cell-source-related activity differences.

Consistent with previous comparisons, MPCs were found to be less osteogenic than MSCs in a non-inflamed setting than MSCs[139]. The above work found lower MPC osteogenic gene expression, ALP activity, and mineralization than MSCs, except for OCN and COL-I gene expression, under normal osteogenic culture. In the presence of IL-1β, MPCs and MSCs preferentially mineralized; this mineralization was not associated with an increase in osteogenic markers (Figure 19). In fact, IL-1β culture generally decreased later osteogenic gene expression and early ALP activity (Figure 22). These findings are consistent with work involving human MSCs reported elsewhere; IL-1β presence between 0.1-10 ng/ml increased culture mineralization

at day 14, later proven to be dependent on ALP activity at that later time point, while inhibiting COL-I and RUNX2 gene expression[225].

The small number of patients available for comparison limits the strength of this study. With two exceptions, *in vitro* age did not affect the stem cell marker expression of tested MPC and MSC populations although neither population exactly matched literature-reported MSC descriptions. Surprisingly, MPCs, though derived from an inflamed environment, exhibited lower HLA-DR expression than MSCs, excluding MPCs from the category of 'activated' MSCs. MPC gained hematopoietic/endothelial[104] CD34 expression with culture time, consistent with reports of an initial $CD34^+$ subpopulation within unsorted muscle-derived cell suspensions[143] and initial differences in CD105 expression, denoting vascular origins[222], between MPC and MSC disappeared by late passage. The presence of $CD34^+$ cells within the MPC population is particularly troubling given the association of $CD34^+$ cells with heterotopic ossification in human genetic disorders. Combined with the work of previous chapters, this work indicates that MPCs must be screened rigorously for their stem cell activities and trophic potential before consideration for use in a patient.

Focusing on non-inflamed comparisons, EC influence on MPC osteogenesis was broadly similar to that reported in the literature for various fibroblast-endothelial systems. Co-culturing of osteoblasts and HUVEC (human umbilical vasculoendothelial cells) resulted in increased osteoblast ALP gene expression; co-culture also suppressed OCN expression, likely mediated by p38 MAPK[227], but this increase could be associated with higher BMP2 gene expression levels in ECs versus MSCs/MPCs[228]. One key difference between the findings reported here from previous findings was a decrease in ALP activity with co-culture when compared to MPC/MSC-culture alone, perhaps due to the use of ECs versus HUVECs or activity normalization (DNA

versus protein)[96]. A global loss of CD31 production and poor gene expression was observed under osteogenic conditions (Figure 20), likely due to long-term culture incompatibilities between MPCs/MSCs and ECs[229]. BMP2 gene expression increased under the influence of both EC and, most strikingly, EC-CM. Increased collagenous matrix deposition under the influence EC-CM suggests a hyper-mineralizing influence of EC-CM[230].

In experiments confirming the effect of inflammation on ECs, moderate changes in EC network forming abilities (Figure 18) were consistent with changes observed with more commonly-used HUVECs[231]. Three inflammation-upregulated angiogenic molecules played significant roles in host immune response and osteogenesis[232,233]. CXCL6 is known to recruit neutrophils, affect EC proliferation, and is produced by fibroblasts upon IL-1β stimulation, but no effects have been reported on osteoblast or MSC differentiation[174,233]. IGF1 regulates the delicate balance between adipogenesis and osteogenesis in MSC and, through its binding with the IGF-receptro (IGFR), increases osteoblast proliferation[234]. IL-1β-induced IL-6 is known to suppress osteoblast activity[90], pressuring differentiated progenitor cells to revert to their stem origins[172]. Taken together, these increases suggested that inflamed ECs might differentially regulate osteogenesis when compared to their non-inflamed counterparts, promoting proliferation and suppressing osteogenic differentiation, depending on the responsiveness of MPCs or MSCs.

EC-influence in the presence of inflammation could not be so easily matched to observations reported elsewhere. EC-CM+I-induced gene expression changes were internally consistent with the hyper-mineralizing phenotype observed using Alizarin Red S staining (Figure 23). Those observations of enhanced MPC-osteogenesis in the presence of EC-CM+I were particularly surprising, given reports of suppression of osteogenesis by inflamed EC through the actions of IL-6; however, one possible explanation for the contrary finding could be the IL-6

stimulated release by MSCs of soluble IL-6 receptor[235,236]. This MPC-upregulation of an anti-inflammatory molecule would make sense if MPCs were primed to dampen inflammation, as has been suggested by *in vitro* work with T-cells[142]. MSCs, perhaps because they were not previously activated, exhibited expected, distinctly lower mature osteogenesis marker expression due to inflamed-EC influence (Figure 24 and Figure 25).

This study faced several limitations. Because such decreases have been observed in MSCs in many previous experiments, this work assumes that gene expression decreases correlated with changes in the cellular microenvironment. Future work would need to confirm functional changes in COL-I deposition and serum OCN and BMP2 concentrations to draw full conclusions about the effect of IL-1β and cell source on osteogenic potential. Additionally, because MSCs from older females were utilized in this study, age- and sex-related differences could explain the observed magnitude differences in OCN gene expression between MPCs and MSCs[81]. The opposite responses to IL-1β of MPCs versus MSCs might be attributed to source-related differences except for the following two observations.

Trauma appears to mobilize an adherent, proliferative, osteogenic cell type ultimately derived from the bone marrow[237], and non-traumatized muscle-derived MPCs were shown to exhibit a strongly mineralizing phenotype, among other phenotypes relevant to heterotopic ossification[238]. In addition, interaction with monocytes and macrophages, among other cell types, was found to alter osteogenesis *in vivo*[239,240]. While the question of ultimate origin of MPCs and their *in vivo* efficacy therefore remain unanswered, this work provides clear evidence for tissue trauma-related differences in the angiogenic- and inflammation-responsive osteogenic potential of the muscle-derived multipotent cells. To gain further insight into the effect of trauma on various multipotent cell activities without cell-sourcing questions, work is ongoing to

compare non-traumatized and blast-traumatized muscle-progenitor cells derived from cadaveric mice. Such a model has the added benefit of recapitulating the precise events thought to initiate the higher incidence of HO observed in blast-traumatized patients[15].

4.5 CONCLUSIONS

Heterotopic ossification affects a significantly larger percentage of blast-traumatized versus non-blast traumatized patients. Work is ongoing to discover a mechanistic reason for this difference, and intense interest has been focused on MPCs in recent years towards that end[6]. Recent work has identified a promising MPC subpopulation that may contribute preferentially to the formation of HO nodules[143]. Despite the substantial characterization work accomplished in the past several years, very scarce data is available about the modulatory capacity of MPCs with respect to other cells, namely T-cells and ECs[139,142], but more importantly, no information has yet been reported on the modulating effect of the wound environment on MPCs. The work presented above is the first to examine cell-cell interactions on MPC osteogenesis.

Taken together, the presented data indicate that when angiogenic and inflammatory stimuli are present, MPCs are more responsive to osteogenic stimuli than MSCs. This work provides evidence that MPCs exhibit a more mature osteogenic phenotype as a direct result of the combination of inflammation and the activities of ECs. Inflammation, in the form of exogenous IL-1β, significantly increases MPC-mineralization but inhibits osteogenic gene expression, while EC influences, both contact-mediated and through soluble factors, seem to promote a more mature, matrix-depositing, osteoblast-phenotype. This is encouraging in the limited context of bone regenerative therapies, but, ultimately worrying from a patient

perspective, as cell migration from an implanted regenerative medicine construct could nucleate heterotopic ossification due to inflammation inherent with normal wound healing. Caution should therefore be applied in developing potential therapies that seek to restore limb skeletal structure or function using MPCs.

5.0 SUMMARY AND FUTURE DIRECTIONS

Under current treatment methods for blast-damaged limbs, the average 24-year-old battlefield-injured military combatant awakens from life-saving surgery to an existence that, at best, requires either expensive prosthetic use or the sub-optimal function and probable pain of a scarred limb. Efforts to improve the function of restored limbs and to understand some of the orthopedic causes of complications are ongoing. Considerable interest has been generated by the idea of using autologous multipotent progenitor/stem cells, particularly MPCs, therapeutically, but as with MSCs, ultimate translation to clinical use remains challenging due to limited understanding of their true and dynamic activity *in vivo*. Within the context of combat-injury-relevant therapies, this work examined the effect of the cellular environment on MPCs' trophic and differentiation abilities.

By varying the physical and cellular environment of MPCs in a context-dependent manner, it was hypothesized that a more thorough understanding of the robustness of regeneration- and clinically-relevant MPC-trophic and -functional activities would be obtained. The preceding chapters detailed attempts to describe MPCs and their therapeutic potential in the context of communications with other cell types, mainly ECs. The significant advantage of easy isolation and autologous sourcing of MPCs, as well as their angiogenic and neurotrophic effects, while promoting their potential therapeutic use, is counterbalanced by increasing evidence that

MPCs readily present a mineralizing phenotype under some of the same influences that were found to enhance MPC context-dependent trophic support.

In the second chapter, which dealt with angiogenesis, it was found that MPCs preferentially promote angiogenesis in a manner distinct from and possibly superior to MSCs. This effect was likely due to MPC-secreted VEGF attracting ECs. MPCs are therefore likely to be useful in the recruitment of vasculature necessary to support an implanted construct. If encapsulated within a degradable hydrogel, viable and autologous MPCs could conceivably be used to facilitate any process requiring enhanced vascular support, including soft tissue or bone reconstruction in blast-traumatized limbs. Future investigations of MPC therapeutic potential as related to MPC angiogenic potential could examine adult animal models of vessel recruitment, as the CAM model involves extremely dynamic, embryonic vessels primed to form new blood vessels.

In the third chapter, the described work added to the knowledge of neurotrophic functions of MPCs by investigating VEGF as an important MPC-produced neurotrophic factor. Distinctly higher VEGF production was seen in MPCs upon neurotrophic induction versus normal growth culture, and distinctly higher neurotrophic activity of MPCs was observed when their influence (either cell contact-mediated or through CM) was combined with that of ECs; together, both cell types preferentially supported the neurite extension of chick embryonic DRG, seeded on aligned, nanofibrous PCL nerve guide conduit and on tissue culture plastic. Selective removal of VEGF revealed a distinct but insignificant dependence of neurite extension on MPC-CM. This suggests that future conduit-based nerve therapies might best support nerve growth by encouraging the growth of neurovascular units versus nerves, alone. This hypothesis could be explored further

using MPCs implanted in a nerve guide conduit to support this strategy, but optimization of the scaffold and its effects on MPC neurotrophic function are still needed.

One possible mechanism to enhance neurotrophic cell-biomaterial interactions and neurotrophic support could be to coat the PCL fibers with ECM molecules. Laminin, collagen, and hyaluronic acid have all been shown to enhance nerve growth[241]. Pilot studies utilizing collagen and collagen-hyaluronic acid coatings on PCL fibers showed promising enhancement of neurite outgrowth without MPC influence, and when encapsulated within hyaluronic acid inside PCL conduits, MPCs and ECs appeared to exhibit increased neurotrophic molecule production and survival when cultured in combination than when cultured alone.

The fourth chapter detailed work that ultimately suggests caution when approaching clinical, therapeutic use of MPCs. To summarize these findings, the osteogenic activities of MPCs in the context of a healing wound bed were explored, and MPCs were found to be a uniquely responsive mineralizing cell population. Early inflammatory markers, in the form of exogenous IL-1β, significantly increased MPC-mineralization but inhibited osteogenic gene expression, while EC influences, via contact-mediated and soluble factor, promoted a more mature osteoblast-phenotype capable of bony matrix deposition. Work in the fourth chapter suggested that this hyper-mineralizing phenotype might be due to the presence in MPCs of endothelial progenitor cells, which have been shown to positively influence fibroblast and MSC bone-forming abilities[242]. Given the intimate association between inflammation and wound healing, these findings suggest that any therapies involving MPCs would need to be tightly monitored for ectopic bone formation. Caution should therefore be applied in developing potential therapies, especially those that seek to restore skeletal tissue structure or function using MPCs.

The investigations presented here further solidify the view of MPCs as a trophic fibroblast with strong mineralizing potential. While primary isolates are known to be heterogeneous unless expanded unequivocally from a single cell progenitor[243], evidence from this work and others indicates that MPCs represent an especially heterogeneous and dynamic mixture of plastic-adherent cells[143]. Clear differences were observed between MPC and MSC osteogenic and angiogenic activities, suggesting several areas of exploration. MPCs' potent effects on and sensitivity to EC influence indicate that MPCs or a significant portion, likely the $CD34^+$ faction, are highly-tuned to EC-derived signals. In particular, MPCs' propensity to form bone in the presence of inflammation and angiogenic cell paracrine influence further solidifies a likely role in heterotopic ossification. Future work should investigate this role by examining the effect of transient versus chronic exposure to inflammation on the osteogenic phenotype of MPCs.

This and other work have shown similarities and clear differences between MPCs and MSCs. While these differences are intriguing from a biological perspective, therapeutic relevance might be more easily found by comparing the different activities of blast-traumatized versus non-traumatized muscle-derived multipotent progenitor cells (MPCs vs. MD-MSCs) since blast-trauma appears to be the cause of distinctive disease pathologies. Work is ongoing to isolate a similar cell population from donor, non-traumatized cadaveric muscle for comparison to MPCs; however, differences in both donor age and muscle state (freshly debrided versus cadaveric) could contribute to unknown variation in MD-MSC activities. If a suitable blast injury mechanism could be introduced, an alternative comparison could be made using an animal model, allowing for direct blast-traumatized versus non-blast-traumatized muscle comparisons; work is ongoing to develop such a system utilizing cadaveric mice.

MPCs have proven to be an interesting study in the dual nature of multipotent cells within a wound environment. On the one hand, MPCs exhibit potent trophic activities, at times displaying superior vascular and sensory nerve support when compared to clinically nutilized MSCs. This support is evident even when MPCs are transferred from standard *in vitro* culture conditions to more clinically relevant constructs. Such therapeutic functions are highly desirable from the standpoint of the severely injured combatant and their medical care team. Mounting evidence, however, cautions against the consideration of MPCs as a combat-derived panacea. Of particular concern is that the chronically inflamed environment of blast–traumatized tissue may quickly restrict the differentiation potential of multipotent cells, and complicate culture-based attempts to direct MPC differentiation and, by extension, MPC-based therapies. This caution is based in part on our findings that additional or continuous exposure of MPCs to IL-1β, simulating an inflammatory environment, results in abnormal mineralization. However, it should be noted that this results from prolonged inflammatory influences. The insights gained from the above work suggest that work in the future should examine the response of MPCs to their environment, specifically the state of tissue inflammation; better control of inflammation is likely to be an important requirement for the development of MPCs for more effective MPC-based therapies with fewer pathological complications.

APPENDIX A

UTILIZED CELL POPULATIONS

Table 4: Cell populations referenced in multiple chapters
MPC1-3 were used for the work described in Chapters 3 and 4 while MPC1-4 were utilized in Chapter 2. MSC3-5 were utilized as control cell types for Chapter4. MSC 1-3 and 5 were utilized for Chapter 3. Initially, MPC derived from 7 patients were available for study. 3 patients were excluded due to low proliferation rates/early senescence by p.5. Upon extended passage, cell senescence in MPC4 was observed at p.8.

***In vivo* cell source**	**ID**	**Isolation age (Years) & gender**	**Passage utilized**
Human blast-traumatized muscle Plastic-adherent cells[134]	MPC1	Individual patient data not available Total population description: 24.4 ± 4, male	3-10
	MPC2		3-10
	MPC3		3-10
	MPC4		3-8
Bone marrow from femoral heads following arthroscopy Plastic-adherent[244]	MSC1*	20, male	3-10
	MSC2*	20, male	3-10
	MSC3*	56, female	3-10
	MSC4*	60, male	3-8
	MSC5*	78, female	3-10
Human foreskin fibroblasts [Lonza]	HFF	Neonates, male	5-8
Human adult dermal microvascular endothelial cells [CDC][183,184]	EC	Immortalized cell line, male	5-8

*Differentiation potential confirmed with *in vitro* adipogenic, osteogenic, and chondrogenic differentiation and colony-forming unit tests.

APPENDIX B

GROWTH FACTOR RELEASE FROM METHACRYLATED-GELATIN CONSTRUCTS

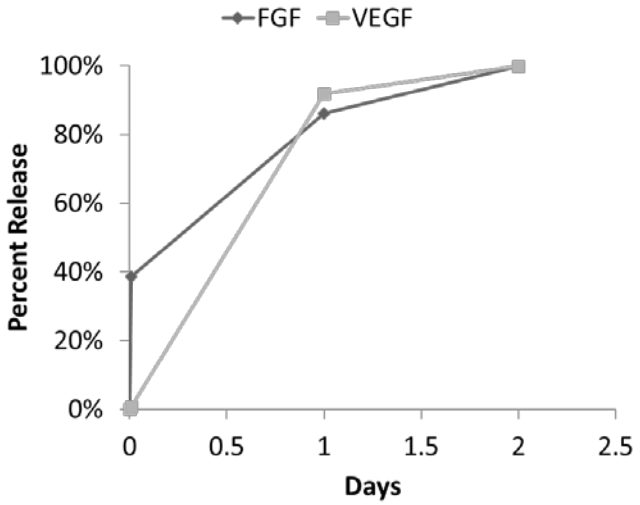

Figure 27: Growth factor release from photocrosslinked gelatin constructs
Encapsulated FGF-2 and VEGF were readily released from the methacrylated gelatin constructs; this implies that cell-produced growth factors would also be rapidly released into the surrounding medium or tissue structures.

BIBLIOGRAPHY

1. Simeone, G. Modern warfare. *An. Med. Publica* **3,** 11–26

2. Jackson, W. M., Nesti, L. J. & Tuan, R. S. Concise review: clinical translation of wound healing therapies based on mesenchymal stem cells. *Stem Cells Transl. Med.* **1,** 44–50 (2012).

3. Fossett, E. & Khan, W. S. Optimising human mesenchymal stem cell numbers for clinical application: a literature review. *Stem Cells Int.* **2012,** 465259 (2012).

4. Leland, A. & Oboroceanu, M.-J. American war and military operations casualties: Lists and statistics. *Congr. Res. Serv.* (2010).

5. Nesti, L. J. *et al.* Differentiation potential of multipotent progenitor cells derived from war-traumatized muscle tissue. *J Bone Jt. Surg Am* **90,** 2390–2398 (2008).

6. Christopherson, G. T. & Nesti, L. J. Stem cell applications in military medicine. *Stem Cell Res. Ther.* **2,** 40 (2011).

7. Tintle, S. M. *et al.* Reoperation after combat-related major lower extremity amputations. *J. Orthop. Trauma* **28,** 232–7 (2014).

8. Langer, V. Management of major limb injuries. *Sci. World J.* **2014,** 640430 (2014).

9. Breidenbach, W. C. Emergency free tissue transfer for reconstruction of acute upper extremity wounds. *Clin. Plast. Surg.* **16,** 505–14 (1989).

10. Tintle, S. M., Baechler, M. F., Nanos, G. P., Forsberg, J. A. & Potter, B. K. Reoperations following combat-related upper-extremity amputations. *J. Bone Joint Surg. Am.* **94,** e1191–6 (2012).

11. Melcer, T., Sechriest, V. F., Walker, J. & Galarneau, M. A comparison of health outcomes for combat amputee and limb salvage patients injured in Iraq and Afghanistan wars. *J. Trauma Acute Care Surg.* **75,** S247–54 (2013).

12. Doukas, W. C. *et al.* The Military Extremity Trauma Amputation/Limb Salvage (METALS) study: outcomes of amputation versus limb salvage following major lower-extremity trauma. *J. Bone Joint Surg. Am.* **95,** 138–45 (2013).

13. Cross, J. D., Ficke, J. R., Hsu, J. R., Masini, B. D. & Wenke, J. C. Battlefield orthopaedic injuries cause the majority of long-term disabilities. *J Am Acad Orthop Surg* **19 Suppl 1,** S1–7 (2011).

14. Andersen, R. C., D'Alleyrand, J.-C. G., Swiontkowski, M. F. & Ficke, J. R. Extremity War Injuries VIII: sequelae of combat injuries. *J. Am. Acad. Orthop. Surg.* **22,** 57–62 (2014).

15. Bharmal, H. M. *et al.* Insights into posttraumatic heterotopic ossification in extremity war injuries. *Curr. Orthop. Pract.* **24,** 127–133 (2013).

16. Potter, B. K., Burns, T. C., Lacap, A. P., Granville, R. R. & Gajewski, D. A. Heterotopic ossification following traumatic and combat-related amputations. Prevalence, risk factors, and preliminary results of excision. *J. Bone Joint Surg. Am.* **89,** 476–86 (2007).

17. Whyte, M. P. *et al.* Fibrodysplasia ossificans progressiva: middle-age onset of heterotopic ossification from a unique missense mutation (c.974G>C, p.G325A) in ACVR1. *J. Bone Miner. Res.* **27,** 729–37 (2012).

18. Reinke, J. M. & Sorg, H. Wound repair and regeneration. *Eur. Surg. Res.* **49,** 35–43 (2012).

19. Jackson, W. M., Nesti, L. J. & Tuan, R. S. Mesenchymal stem cell therapy for attenuation of scar formation during wound healing. *Stem Cell Res. Ther.* **3,** 20 (2012).

20. Martin, P. Wound healing - aiming for perfect skin regeneration. **276,** (1997).

21. Rodrigues, M., Turner, O., Stolz, D., Griffith, L. G. & Wells, A. Production of reactive oxygen species by multipotent stromal cells/mesenchymal stem cells upon exposure to fas ligand. *Cell Transplant.* **21,** 2171–87 (2012).

22. Eming, S. A., Krieg, T. & Davidson, J. M. Inflammation in wound repair: molecular and cellular mechanisms. *J. Invest. Dermatol.* **127,** 514–25 (2007).

23. Hsu, A. & Mustoe, T. a. The Principles of Wound Healing. *Plast. Surg. Secrets* 3–7 (2010). doi:10.1016/B978-0-323-03470-8.00001-6

24. Ball, S. G., Shuttleworth, C. A. & Kielty, C. M. Mesenchymal stem cells and neovascularization: role of platelet-derived growth factor receptors. *J. Cell. Mol. Med.* **11,** 1012–30 (2007).

25. Potente, M., Gerhardt, H. & Carmeliet, P. Basic and therapeutic aspects of angiogenesis. *Cell* **146,** 873–87 (2011).

26. Weis, S. M. & Cheresh, D. A. Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* **437,** 497–504 (2005).

27. Carmeliet, P. & Jain, R. K. Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473,** 298–307 (2011).

28. Birbrair, A. *et al.* Pericytes: multitasking cells in the regeneration of injured, diseased, and aged skeletal muscle. *Front. Aging Neurosci.* **6,** 1–32 (2014).

29. Chauvet, S., Burk, K. & Mann, F. Navigation rules for vessels and neurons: cooperative signaling between VEGF and neural guidance cues. *Cell. Mol. Life Sci.* **70,** 1685–703 (2013).

30. Stegen, S., van Gastel, N. & Carmeliet, G. Bringing new life to damaged bone: The importance of angiogenesis in bone repair and regeneration. *Bone* **70,** 19–27 (2014).

31. Iruela-Arispe, M. L. & Dvorak, H. F. Angiogenesis: a dynamic balance of stimulators and inhibitors. *Thromb. Haemost.* **78,** 672–7 (1997).

32. Licht, T. & Keshet, E. Delineating multiple functions of VEGF-A in the adult brain. *Cell. Mol. Life Sci.* **70,** 1727–37 (2013).

33. Yang, X. & Cepko, C. L. Flk-1, a receptor for vascular endothelial growth factor (VEGF), is expressed by retinal progenitor cells. *J. Neurosci.* **16,** 6089–6099 (1996).

34. Takagi, S. *et al.* Expression of a cell adhesion molecule, neuropilin, in the developing chick nervous system. *Dev. Biol.* **170,** 207–22 (1995).

35. Shibuya, M. Vascular endothelial growth factor and its receptor system: physiological functions in angiogenesis and pathological roles in various diseases. *J. Biochem.* **153,** 13–9 (2013).

36. Goel, H. L. & Mercurio, A. M. VEGF targets the tumour cell. *Nat. Rev. Cancer* **13,** 871–82 (2013).

37. Goodwin, A. M. In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. *Microvasc. Res.* **74,** 172–83 (2007).

38. Kubota, Y., Kleinman, H. K., Martin, G. R. & Lawley, T. J. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J. Cell Biol.* **107,** 1589–98 (1988).

39. Yarrow, J. C., Perlman, Z. E., Westwood, N. J. & Mitchison, T. J. A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods. *BMC Biotechnol.* **4,** 21 (2004).

40. Boyden, S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. Exp. Med.* **115,** 453–66 (1962).

41. Eccles, S. A., Box, C. & Court, W. Cell migration/invasion assays and their application in cancer drug discovery. *Biotechnol. Annu. Rev.* **11,** 391–421 (2005).

42. Van Hinsbergh, V. W. M., Engelse, M. A. & Quax, P. H. A. Pericellular proteases in angiogenesis and vasculogenesis. *Arterioscler. Thromb. Vasc. Biol.* **26,** 716–28 (2006).

43. Yalcin, H. C., Shekhar, A., Rane, A. A. & Butcher, J. T. An ex-ovo chicken embryo culture system suitable for imaging and microsurgery applications. *J. Vis. Exp.* e2154 (2010). doi:10.3791/2154

44. Norrby, K. In vivo models of angiogenesis. *J. Cell. Mol. Med.* **10,** 588–612 (2006).

45. Vu, M. T., Smith, C. F., Burger, P. C. & Klintworth, G. K. Methods in laboratory investigation. An evaluation of methods to quantitate the chick chorioallantoic membrane assay in angiogenesis. *Lab. Investig.* **53,** 499–508 (1985).

46. Fellah, J. S., Jaffredo, T., Nagy, N. & Dunon, D. *Avian Immunology*. *Avian Immunol.* (Elsevier, 2014). doi:10.1016/B978-0-12-396965-1.00003-0

47. Schomann, T., Qunneis, F., Widera, D., Kaltschmidt, C. & Kaltschmidt, B. Improved method for ex ovo-cultivation of developing chicken embryos for human stem cell xenografts. *Stem Cells Int.* **2013,** 960958 (2013).

48. Lundborg, G. *Nerve Injury and Repair*. (Churchill Livingstone, 1988).

49. Neufeld, G., Cohen, T., Gengrinovitch, S. & Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J.* **13,** 9–22 (1999).

50. Uniewicz, K. A., Cross, M. J. & Fernig, D. G. Exogenous recombinant dimeric neuropilin-1 is sufficient to drive angiogenesis. *J. Biol. Chem.* **286,** 12–23 (2011).

51. Griffin, J. W., Hogan, M. V, Chhabra, A. B. & Deal, D. N. Peripheral nerve repair and reconstruction. *J Bone Jt. Surg Am* **95,** 2144–2151 (2013).

52. Novak, C. B., Anastakis, D. J., Beaton, D. E. & Katz, J. Patient-reported outcome after peripheral nerve injury. *J Hand Surg Am* **34,** 281–287 (2009).

53. Novak, C. B., Anastakis, D. J., Beaton, D. E. & Katz, J. Evaluation of pain measurement practices and opinions of peripheral nerve surgeons. *Hand (N Y)* **4,** 344–349 (2009).

54. RIVERA, J. C., GLEBUS, G. P. & CHO, M. S. Disability following combat-sustained nerve injury of the upper limb. *Bone Jt. J* **96-B,** 254–258 (2014).

55. Visser, P. A., Hermreck, A. S., Pierce, G. E., Thomas, J. H. & Hardin, C. A. Prognosis of nerve injuries incurred during acute trauma to peripheral arteries. *Am. J. Surg.* **140,** 596–9 (1980).

56. Seddon, H. J. A Classification of Nerve Injuries. *Br. Med. J.* **2,** 237–239 (1942).

57. Sunderland, S. A classification of peripheral nerve injuries producing loss of function. *Brain* **74,** 491–516 (1951).

58. Seckel, B. R. Enhancement of peripheral nerve regeneration. *Muscle Nerve* **13,** 785–800 (1990).

59. Belkas, J. S., Shoichet, M. S. & Midha, R. Peripheral nerve regeneration through guidance tubes. *Neurol. Res.* **26,** 151–60 (2004).

60. Daly, W., Yao, L., Zeugolis, D., Windebank, A. & Pandit, A. A biomaterials approach to peripheral nerve regeneration: bridging the peripheral nerve gap and enhancing functional recovery. *J R Soc Interface* **9,** 202–221 (2012).

61. Rotshenker, S. Wallerian degeneration: the innate-immune response to traumatic nerve injury. *J. Neuroinflammation* **8,** 109 (2011).

62. Brushart, T. *Nerve Repair*. (2011). doi:10.1093/med/9780195169904.001.0001

63. Nectow, A. R., Marra, K. G. & Kaplan, D. L. Biomaterials for the development of peripheral nerve guidance conduits. *Tissue Eng. Part B. Rev.* **18,** 40–50 (2012).

64. Fu, S. Y. & Gordon, T. Contributing factors to poor functional recovery after delayed nerve repair: prolonged denervation. *J. Neurosci.* **15,** 3886–3895 (1995).

65. Kingham, P. J. & Terenghi, G. Bioengineered nerve regeneration and muscle reinnervation. *J Anat* **209,** 511–526 (2006).

66. Jin, K., Mao, X. O. & Greenberg, D. a. Vascular endothelial growth factor stimulates neurite outgrowth from cerebral cortical neurons via Rho kinase signaling. *J. Neurobiol.* **66,** 236–42 (2006).

67. Allodi, I., Udina, E. & Navarro, X. Specificity of peripheral nerve regeneration: Interactions at the axon level. *Prog. Neurobiol.* **98,** 16–37 (2012).

68. Deumens, R. *et al.* Repairing injured peripheral nerves: Bridging the gap. *Prog Neurobiol* **92,** 245–276 (2010).

69. Joyce, N. *et al.* Mesenchymal stem cells for the treatment of neurodegenerative disease. *Regen. Med.* **5,** 933–946 (2010).

70. Lee, S. K. & Wolfe, S. W. Peripheral nerve injury and repair. *J. Am. Acad. Orthop. Surg.* **8,** 243–252 (1999).

71. Kehoe, S., Zhang, X. F. & Boyd, D. FDA approved guidance conduits and wraps for peripheral nerve injury: A review of materials and efficacy. *Injury* **43,** 553–572 (2012).

72. Arslantunali, D. Peripheral nerve conduits : technology update. *Med. devices Evid. Res.* **7,** 405–424 (2014).

73. Nikolaev, S. I., Gallyamov, a R., Mamin, G. V & Chelyshev, Y. a. Poly(ε-caprolactone) nerve conduit and local delivery of vegf and fgf2 genes stimulate neuroregeneration. *Bull. Exp. Biol. Med.* **157,** 155–8 (2014).

74. Young, S., Wong, M., Tabata, Y. & Mikos, A. G. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. *J. Control. Release* **109,** 256–74 (2005).

75. Faroni, A., Mobasseri, S. A., Kingham, P. J. & Reid, A. J. Peripheral nerve regeneration: Experimental strategies and future perspectives. *Adv. Drug Deliv. Rev.* (2014). doi:10.1016/j.addr.2014.11.010

76. Barbarisi, M. *et al.* Use of polycaprolactone (PCL) as scaffolds for the regeneration of nerve tissue. *J. Biomed. Mater. Res. A* (2014). doi:10.1002/jbm.a.35318

77. Cao, H., Liu, T. & Chew, S. Y. The application of nanofibrous scaffolds in neural tissue engineering. *Adv. Drug Deliv. Rev.* **61,** 1055–1064 (2009).

78. Fan, L., Yu, Z., Li, J., Dang, X. & Wang, K. Schwann-like cells seeded in acellular nerve grafts improve nerve regeneration. *BMC Musculoskelet. Disord.* **15,** 165 (2014).

79. Wu, Y. *et al.* Effects of vascular endothelial cells on osteogenic differentiation of noncontact co-cultured periodontal ligament stem cells under hypoxia. *J. Periodontal Res.* **48,** 52–65 (2013).

80. Kanczler, J. M. & Oreffo, R. O. C. Osteogenesis and angiogenesis: The potential for engineering bone. *Eur. Cells Mater.* **15,** 100–114 (2008).

81. Del Pino, J. *et al.* Influence of sex, age, and menopause in serum osteocalcin (BGP) levels. *Klin. Wochenschr.* **69,** 1135–1138 (1990).

82. Shrivats, A. R. & Hollinger, J. O. in *Biomimetics Stem Cells* (Vunjak-Novakovic, G. & Turksen, K.) 151 (Humana Press, 2014).

83. Carreira, A. C., Alves, G. G., Zambuzzi, W. F., Sogayar, M. C. & Granjeiro, J. M. Bone Morphogenetic Proteins: Structure, biological function and therapeutic applications. *Arch. Biochem. Biophys.* **561C,** 64–73 (2014).

84. Tsuchida, K. *et al.* Activin signaling as an emerging target for therapeutic interventions. *Cell Commun. Signal.* **7,** 15 (2009).

85. Gaddy-Kurten, D., Coker, J. K., Abe, E., Jilka, R. L. & Manolagas, S. C. Inhibin Suppresses and Activin Stimulates Osteoblastogenesis and Osteoclastogenesis in Murine Bone Marrow Cultures. *Endocrinology* (2013).

86. Rosset, P., Deschaseaux, F. & Layrolle, P. Cell therapy for bone repair. *Orthop. Traumatol. Surg. Res.* **100,** S107–12 (2014).

87. Liu, R. *et al.* Myoblast sensitivity and fibroblast insensitivity to osteogenic conversion by BMP-2 correlates with the expression of Bmpr-1a. *BMC Musculoskelet. Disord.* **10,** 51 (2009).

88. Vukicevic, S. *et al.* The clinical use of bone morphogenetic proteins revisited: a novel biocompatible carrier device OSTEOGROW for bone healing. *Int. Orthop.* **38,** 635–47 (2014).

89. Twine, N. a., Chen, L., Pang, C. N., Wilkins, M. R. & Kassem, M. Identification of differentiation-stage specific markers that define the ex vivo osteoblastic phenotype. *Bone* **67,** 23–32 (2014).

90. Haynesworth, S. E., Baber, M. A. & Caplan, A. I. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha. *J. Cell. Physiol.* **166,** 585–92 (1996).

91. Kuroki, T., Shingu, M., Koshihara, Y. & Nobunaga, M. Effects of cytokines on alkaline phosphatase and osteocalcin production, calcification and calcium release by human osteoblastic cells. *Br. J. Rheumatol.* **33,** 224–230 (1994).

92. FLEISCH, H., RUSSELL, R. G. G. & STRAUMANN, F. Effect of Pyrophosphate on Hydroxyapatite and Its Implications in Calcium Homeostasis. *Nature* **212,** 901–903 (1966).

93. Sidney, L. E., Kirkham, G. R. & Buttery, L. D. Comparison of osteogenic differentiation of embryonic stem cells and primary osteoblasts revealed by responses to IL-1β, TNF-α, and IFN-γ. *Stem Cells Dev.* **23,** 605–17 (2014).

94. Bellows, C. G., Heersche, J. N. M. & Aubin, J. E. Inorganic phosphate added exogenously or released from β-glycerophosphate initiates mineralization of osteoid nodules in vitro. *Bone Miner.* **17,** 15–29 (1992).

95. Salem, H. K. & Thiemermann, C. Mesenchymal stromal cells: Current understanding and clinical status. *Stem Cells* **28,** 585–596 (2010).

96. Villars, F., Bordenave, L., Bareille, R. & Amédée, J. Effect of human endothelial cells on human bone marrow stromal cell phenotype: role of VEGF? *J. Cell. Biochem.* **79,** 672–85 (2000).

97. Lin, F.-H., Chang, J. B., McGuire, M. H., Yee, J. a & Brigman, B. E. Biphasic effects of interleukin-1beta on osteoblast differentiation in vitro. *J. Orthop. Res.* **28,** 958–64 (2010).

98. Ferreira, E. *et al.* Inflammatory cytokines induce a unique mineralizing phenotype in mesenchymal stem cells derived from human bone marrow. *J. Biol. Chem.* **288,** 29494–29505 (2013).

99. Kuhn, N. Z. & Tuan, R. S. Regulation of stemness and stem cell niche of mesenchymal stem cells: implications in tumorigenesis and metastasis. *J. Cell. Physiol.* **222,** 268–77 (2010).

100. Spiropoulos, A. *et al.* Rapid clinical-scale propagation of mesenchymal stem cells using cultures initiated with immunoselected bone marrow CD105+ cells. *J. Cell. Mol. Med.* **15,** 1983–8 (2011).

101. Parekkadan, B. & Milwid, J. M. Mesenchymal stem cells as therapeutics. **12,** 87–117 (2010).

102. Lau, T. T. & Wang, D.-A. Stromal cell-derived factor-1 (SDF-1): Homing factor for engineered regenerative medicine. *Expert Opin. Biol. Ther.* **11,** 189–197 (2011).

103. Murray, I. R. *et al.* Natural history of mesenchymal stem cells, from vessel walls to culture vessels. *Cell. Mol. Life Sci.* **71,** 1353–74 (2014).

104. Dominici, M. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8,** 315–317 (2006).

105. Kolf, C. M., Cho, E. & Tuan, R. S. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res. Ther.* **9,** 204 (2007).

106. Caddick, J., Kingham, P. J., Gardiner, N. J., Wiberg, M. & Terenghi, G. Phenotypic and functional characteristics of mesenchymal stem cells differentiated along a Schwann cell lineage. *Glia* **54,** 840–9 (2006).

107. Caplan, A. I. Why are MSCs therapeutic ? New data : new insight. 318–324 (2009). doi:10.1002/path

108. Aurora, A. B. & Olson, E. N. Immune modulation of stem cells and regeneration. *Cell Stem Cell* **15,** 14–25 (2014).

109. Aggarwal, S. & Pittenger, M. F. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* **105,** 1815–1822 (2005).

110. Jacobs, S. a, Roobrouck, V. D., Verfaillie, C. M. & Van Gool, S. W. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. *Immunol. Cell Biol.* **91,** 32–9 (2013).

111. Ankrum, J. a, Ong, J. F. & Karp, J. M. Mesenchymal stem cells: immune evasive, not immune privileged. *Nat. Biotechnol.* **32,** 252–60 (2014).

112. Friedenstein, A. J., Piatetzky-Shapiro, I. I. & Petrakova, K. V. Osteogenesis in transplants of bone marrow cells. *J. Embryol. Exp. Morphol.* **16,** 381–390 (1966).

113. Owen, M. The origin of bone cells. *Int. Rev. Cytol.* **28,** 213–238 (1970).

114. Bianco, P. Stem cells and bone: A historical perspective. *Bone* **70,** 2–9 (2015).

115. Jackson, W. M., Nesti, L. J. & Tuan, R. S. Potential therapeutic applications of muscle-derived mesenchymal stem and progenitor cells. *Expert Opin. Biol. Ther.* **10,** 505–17 (2010).

116. Nuschke, A. Activity of mesenchymal stem cells in therapies for chronic skin wound healing. *Organogenesis* **10,** 29–37 (2014).

117. Waterman, R. S., Tomchuck, S. L., Henkle, S. L. & Betancourt, A. M. A new mesenchymal stem cell (MSC) paradigm: Polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS One* **5,** (2010).

118. Joo, Y. O. *et al.* The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. *Stem Cells* **26,** 1047–1055 (2008).

119. Watson, L., Elliman, S. J. & Coleman, C. M. From isolation to implantation: a concise review of mesenchymal stem cell therapy in bone fracture repair. *Stem Cell Res. Ther.* **5,** 51 (2014).

120. Kokai, L. E., Marra, K. & Rubin, J. P. Adipose stem cells: Biology and clinical applications for tissue repair and regeneration. *Transl. Res.* **163,** 399–408 (2014).

121. Gottipamula, S. *et al.* Isolation, expansion and characterization of bone marrow-derived mesenchymal stromal cells in serum-free conditions. *Cell Tissue Res.* **356,** 123–135 (2014).

122. Marconi, S. *et al.* Human adipose-derived mesenchymal stem cells systemically injected promote peripheral nerve regeneration in the mouse model of sciatic crush. *Tissue Eng. Part A* **18,** 1264–72 (2012).

123. Hass, R., Kasper, C., Böhm, S. & Jacobs, R. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun. Signal.* **9,** (2011).

124. Mesimäki, K. *et al.* Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. *Int. J. Oral Maxillofac. Surg.* **38,** 201–9 (2009).

125. Lavasani, M. *et al.* Human muscle – derived stem / progenitor cells promote functional murine peripheral nerve regeneration. **124,** (2014).

126. Chen, W. C. W. *et al.* Cellular kinetics of perivascular MSC precursors. *Stem Cells Int.* **2013,** (2013).

127. Qu, Z. *et al.* Development of approaches to improve cell survival in myoblast transfer therapy. *J. Cell Biol.* **142,** 1257–1267 (1998).

128. Gao, X. *et al.* A comparison of bone regeneration with human mesenchymal stem cells and muscle-derived stem cells and the critical role of BMP. *Biomaterials* **35,** 6859–70 (2014).

129. Huard, J., Gharaibeh, B. & Usas, A. Regenerative Medicine Based on Muscle-Derived Stem Cells. *Oper. Tech. Orthop.* **20,** 119–126 (2010).

130. Birbrair, A. *et al.* Skeletal muscle pericyte subtypes differ in their differentiation potential. *Stem Cell Res.* **10,** 67–84 (2013).

131. Birbrair, A. *et al.* Type-2 pericytes participate in normal and tumoral angiogenesis. *Am. J. Physiol. Cell Physiol.* **307,** C25–38 (2014).

132. Bischoff, R. A satellite cell mitogen from crushed adult muscle. *Dev. Biol.* **115,** 140–147 (1986).

133. Intrinsic and therapeutic factors determining the recovery of motor function after peripheral nerve transection. *Ann. Anat.* **193,** 286–303 (2011).

134. Jackson, W. M., Aragon, A. B., Bulken-Hoover, J. D., Nesti, L. J. & Tuan, R. S. Putative heterotopic ossification progenitor cells derived from traumatized muscle. *J Orthop Res* **27,** 1645–1651 (2009).

135. Jackson, W. M. *et al.* Cytokine expression in muscle following traumatic injury. *J. Orthop. Res.* **29,** 1613–1620 (2011).

136. Ji, Y. *et al.* Heterotopic ossification following musculoskeletal trauma: modeling stem and progenitor cells in their microenvironment. *Adv. Exp. Med. Biol.* **720,** 39–50 (2011).

137. Pretheeban, T., Lemos, D. R., Paylor, B., Zhang, R.-H. & Rossi, F. M. Role of stem/progenitor cells in reparative disorders. *Fibrogenesis Tissue Repair* **5,** 20 (2012).

138. Titorencu, I., Pruna, V., Jinga, V. V & Simionescu, M. Osteoblast ontogeny and implications for bone pathology: an overview. *Cell Tissue Res.* **355,** 23–33 (2014).

139. Jackson, W. M. *et al.* Differentiation and regeneration potential of mesenchymal progenitor cells derived from traumatized muscle tissue. *J. Cell. Mol. Med.* **15,** 2377–88 (2011).

140. Jackson, W. M. *et al.* Mesenchymal progenitor cells derived from traumatized muscle enhance neurite growth. *J. Tissue Eng. Regen. Med.* **7,** 443–51 (2013).

141. Bulken-Hoover, J. D. *et al.* Inducible expression of neurotrophic factors by mesenchymal progenitor cells derived from traumatically injured human muscle. *Mol Biotechnol* **51,** 128–136 (2012).

142. Djouad, F. *et al.* Activin A expression regulates multipotency of mesenchymal progenitor cells. *Stem Cell Res. Ther.* **1,** 11 (2010).

143. Woodard, G. E. *et al.* Characterization of Discrete Subpopulations of Progenitor Cells in Traumatic Human Extremity Wounds. *PLoS One* **9,** e114318 (2014).

144. Santibanez, J. F. & Kocic, J. Transforming growth factor-β superfamily, implications in development and differentiation of stem cells. *Biomol. Concepts* **3,** 429–445 (2012).

145. Scrivo, R., Vasile, M., Bartosiewicz, I. & Valesini, G. Inflammation as 'common soil' of the multifactorial diseases. *Autoimmun. Rev.* **10,** 369–74 (2011).

146. Hsu, Y.-C., Chen, S.-L., Wang, D.-Y. & Chiu, I.-M. Stem Cell-Based Therapy in Neural Repair. *Arch. Biochem. Biophys.* **36,** 98–105 (2013).

147. Mahdi, N. S. & Rahbarghazi, R. Interactions of Mesenchymal Stem Cells with Endothelial Cells. (2014).

148. Henry, T. D. The VIVA Trial: Vascular Endothelial Growth Factor in Ischemia for Vascular Angiogenesis. *Circulation* **107,** 1359–1365 (2003).

149. Zhou, Y. F. *et al.* Effects of gene delivery on collateral development in chronic hypoperfusion: diverse effects of angiopoietin-1 versus vascular endothelial growth factor. *J. Am. Coll. Cardiol.* **44,** 897–903 (2004).

150. Chae, J. K. *et al.* Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. *Arterioscler. Thromb. Vasc. Biol.* **20,** 2573–8 (2000).

151. Cao, R. *et al.* Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nat. Med.* **9,** 604–13 (2003).

152. Zachary, I., Mathur, A., Yla-Herttuala, S. & Martin, J. Vascular protection: A novel nonangiogenic cardiovascular role for vascular endothelial growth factor. *Arterioscler. Thromb. Vasc. Biol.* **20,** 1512–1520 (2000).

153. Gkaliagkousi, E. Nitric oxide signalling in the regulation of cardiovascular and platelet function. *Front. Biosci.* **16,** 1873 (2011).

154. Murakami, M. Signaling required for blood vessel maintenance: molecular basis and pathological manifestations. *Int. J. Vasc. Med.* **2012,** 293641 (2012).

155. Hung, S.-C., Pochampally, R. R., Chen, S.-C., Hsu, S.-C. & Prockop, D. J. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K-Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells* **25,** 2363–70 (2007).

156. Oskowitz, A., McFerrin, H., Gutschow, M., Carter, M. L. & Pochampally, R. Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic. *Stem Cell Res.* **6,** 215–25 (2011).

157. Ranganath, S. H., Levy, O., Inamdar, M. S. & Karp, J. M. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell Stem Cell* **10,** 244–58 (2012).

158. Roche, S. *et al.* Comparative analysis of protein expression of three stem cell populations: models of cytokine delivery system in vivo. *Int. J. Pharm.* **440,** 72–82 (2013).

159. Jiang, B., Rue, E., Wang, G., Roe, R. & Semenza, G. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J. Biol. Chem.* **271,** 17771–8 (1996).

160. Lozito, T. P., Jackson, W. M., Nesti, L. J. & Tuan, R. S. Human mesenchymal stem cells generate a distinct pericellular zone of MMP activities via binding of MMPs and secretion of high levels of TIMPs. *Matrix Biol.* **34,** 132–43 (2014).

161. Kinnaird, T. *et al.* Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* **109,** 1543–9 (2004).

162. Kinnaird, T. *et al.* Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ. Res.* **94,** 678–85 (2004).

163. Jackson, W. M. *et al.* Mesenchymal progenitor cells derived from traumatized human muscle. *J Tissue Eng Regen Med* **3,** 129–138 (2009).

164. Yagi, H., Tan, J. & Tuan, R. S. Polyphenols suppress hydrogen peroxide-induced oxidative stress in human bone-marrow derived mesenchymal stem cells. *J. Cell. Biochem.* **114,** 1163–73 (2013).

165. Carpentier, G., Martinelli, M., Courty, J. & Cascone, I. Angiogenesis Analyzer for ImageJ. in *4th ImageJ User Dev. Conf. Proc.* 198–201 (2012).

166. Lin, H. *et al.* Application of visible light-based projection stereolithography for live cell-scaffold fabrication with designed architecture. *Biomaterials* **34,** 331–339 (2013).

167. Dunn, B. E. & Fitzharris, T. P. Differentiation of the chorionic epithelium of chick embryos maintained in shell-less culture. *Dev. Biol.* **71,** 216–227 (1979).

168. Lozito, T. P. *et al.* Three-dimensional osteochondral microtissue to model pathogenesis of osteoarthritis. *Stem Cell Res. Ther.* **4 Suppl 1,** S6 (2013).

169. Lin, H., Cheng, A. W., Alexander, P. G., Beck, A. M. & Tuan, R. S. Cartilage Tissue Engineering Application of Injectable Gelatin Hydrogel with In Situ Visible-Light-Activated Gelation Capability in both Air and Aqueous Solution. *Tissue Eng. Part A* **00,** 1–10 (2014).

170. Ribatti, D. *et al.* New model for the study of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane: the gelatin sponge/ chorioallantoic membrane assay. *J. Vasc Res* **34,** 455–463 (1997).

171. Rosen, E. M., Liu, D., Setter, E., Bhargava, M. & Goldberg, I. D. Interleukin-6 stimulates motility of vascular endothelium. *EXS* **59,** 194–205 (1991).

172. Pricola, K. L., Kuhn, N. Z., Haleem-Smith, H., Song, Y. & Tuan, R. S. Interleukin-6 maintains bone marrow-derived mesenchymal stem cell stemness by an ERK1/2-dependent mechanism. *J Cell Biochem* **108,** 577–588 (2009).

173. Ho, I. A. *et al.* Human bone marrow-derived mesenchymal stem cells suppress human glioma growth through inhibition of angiogenesis. *Stem Cells* **31,** 146–155 (2013).

174. Lee, J.-K. *et al.* Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS One* **8,** e84256 (2013).

175. Lee, C. *et al.* Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* **126,** 2601–11 (2012).

176. Huang, L. *et al.* Exosomes in Mesenchymal Stem Cells, a New Therapeutic Strategy for Cardiovascular Diseases? *Int. J. Biol. Sci.* **11,** 238–245

177. Cimpean, A. M., Ribatti, D. & Raica, M. A brief history of angiogenesis assays. *Int. J. Dev. Biol.* **55,** 377–382 (2011).

178. Ades, E. W. *et al.* HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J. Invest. Dermatol.* **99,** 683–90 (1992).

179. Hsiao, S. T.-F. *et al.* Comparative analysis of paracrine factor expression in human adult mesenchymal stem cells derived from bone marrow, adipose, and dermal tissue. *Stem Cells Dev.* **21,** 2189–203 (2012).

180. Kachgal, S. & Putnam, A. J. Mesenchymal stem cells from adipose and bone marrow promote angiogenesis via distinct cytokine and protease expression mechanisms. *Angiogenesis* **14,** 47–59 (2011).

181. Ghajar, C. M. *et al.* Mesenchymal cells stimulate capillary morphogenesis via distinct proteolytic mechanisms. *Exp. Cell Res.* **316,** 813–25 (2010).

182. Kundi, S., Bicknell, R. & Ahmed, Z. The role of angiogenic and wound-healing factors after spinal cord injury in mammals. *Neurosci. Res.* **76,** 1–9 (2013).

183. Lozito, T. P., Kuo, C. K., Taboas, J. M. & Tuan, R. S. Human mesenchymal stem cells express vascular cell phenotypes upon interaction with endothelial cell matrix. *J. Cell. Biochem.* **107,** 714–22 (2009).

184. Lozito, T. P., Taboas, J. M., Kuo, C. K. & Tuan, R. S. Mesenchymal stem cell modification of endothelial matrix regulates their vascular differentiation. *J. Cell. Biochem.* **107,** 706–13 (2009).

185. Norman, J. & Desai, T. Methods for Fabrication of Nanoscale Topography for Tissue Engineering Scaffolds. *Ann. Biomed. Eng.* **34,** 89–101 (2006).

186. Kim, Y., Haftel, V. K., Kumar, S. & Bellamkonda, R. V. The role of aligned polymer fiber-based constructs in the bridging of long peripheral nerve gaps. *Biomaterials* **29,** 3117–3127 (2008).

187. Yang, F., Murugan, R., Wang, S. & Ramakrishna, S. Electrospinning of nano/micro scale poly(l-lactic acid) aligned fibers and their potential in neural tissue engineering. *Biomaterials* **26,** 2603–2610 (2005).

188. Li, W.-J., Mauck, R. L., Cooper, J. A., Yuan, X. & Tuan, R. S. Engineering controllable anisotropy in electrospun biodegradable nanofibrous scaffolds for musculoskeletal tissue engineering. *J Biomech* **40,** 1686–1693 (2007).

189. Kokai, L. E., Ghaznavi, A. M. & Marra, K. G. Incorporation of double-walled microspheres into polymer nerve guides for the sustained delivery of glial cell line-derived neurotrophic factor. *Biomaterials* **31,** 2313–2322 (2010).

190. Crigler, L., Robey, R. C., Asawachaicharn, A., Gaupp, D. & Phinney, D. G. Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. *Exp. Neurol.* **198,** 54–64 (2006).

191. Guo, S. & Lo, E. H. Dysfunctional cell-cell signaling in the neurovascular unit as a paradigm for central nervous system disease. *Stroke* **40,** S4–S7 (2009).

192. Mahay, D. *et al.* Growth factors in mesenchymal stem cells following glial-cell differentiation. *Biotechnol. Appl. Biochem.* **51,** 167–76 (2008).

193. Mahay, D., Terenghi, G. & Shawcross, S. G. Schwann cell mediated trophic effects by differentiated mesenchymal stem cells. *Exp. Cell Res.* **314,** 2692–701 (2008).

194. Oliveira, J. T. *et al.* Mesenchymal stem cells in a polycaprolactone conduit enhance median-nerve regeneration, prevent decrease of creatine phosphokinase levels in muscle, and improve functional recovery in mice. *Neuroscience* **170,** 1295–1303 (2010).

195. Hansen, T., Moss, A. & Brindle, N. Vascular Endothelial Growth Factor and Angiopoietins in Neurovascular Regeneration and Protection Following Stroke. *Curr. Neurovasc. Res.* **5,** 236–245 (2008).

196. Sondell, M., Sundler, F. & Kanje, M. Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the ¯ k-1 receptor. *Neuroscience* **12,** 4243–4254 (2000).

197. Pan, Z., Fukuoka, S., Karagianni, N., Guaiquil, V. H. & Rosenblatt, M. I. Vascular endothelial growth factor promotes anatomical and functional recovery of injured peripheral nerves in the avascular cornea. *FASEB J.* 1–12 (2013). doi:10.1096/fj.12-225185

198. Doube, M. *et al.* BoneJ: Free and extensible bone image analysis in ImageJ. *Bone* **47,** 1076–9 (2010).

199. Liu, Z. Q. Scale space approach to directional analysis of images. *Appl. Opt.* **30,** 1369–73 (1991).

200. Preibisch, S., Saalfeld, S. & Tomancak, P. Globally optimal stitching of tiled 3D microscopic image acquisitions. *Bioinformatics* **25,** 1463 (2009).

201. Dezawa, M. *et al.* Bone marrow stromal cells generate muscle cells and repair muscle degeneration. *Science* **309,** 314–317 (2005).

202. Reneker, D. H. & Yarin, A. L. Electrospinning jets and polymer nanofibers. *Polymer (Guildf).* **49,** 2387–2425 (2008).

203. Miyamoto, M., Murphy, T., Schnaar, R. & Coyle, J. Antioxidants protect against glutamate-induced cytotoxicity in a neuronal cell line. *J. Pharmacol. Exp. Ther.* **250,** 1132–1140 (1989).

204. Cameron, N. E., Cotter, M. A. & Maxfield, E. K. Anti-oxidant treatment prevents the development of peripheral nerve dysfunction in streptozotocin-diabetic rats. *Diabetologia* **36,** 299–304 (1993).

205. Pinholt, C., Hartvig, R. A., Medlicott, N. J. & Jorgensen, L. The importance of interfaces in protein drug delivery - why is protein adsorption of interest in pharmaceutical formulations? *Expert Opin. Drug Deliv.* **8,** 949–64 (2011).

206. Jin, K. *et al.* Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11946–11950 (2002).

207. Tamaki, T. Bridging long gap peripheral nerve injury using skeletal muscle-derived multipotent stem cells. *Neural Regen. Res.* **9,** 1333 (2014).

208. Storm, T. *et al.* Chemical activation and changes in surface morphology of poly(ε-caprolactone) modulate VEGF responsiveness of human endothelial cells. *J. Mater. Sci. Mater. Med.* **25,** 2003–15 (2014).

209. Ijichi, A., Sakuma, S. & Tofilon, P. J. Hypoxia-induced vascular endothelial growth factor expression in normal rat astrocyte cultures. *Glia* **14,** 87–93 (1995).

210. Tamaki, T. *et al.* Preferential and comprehensive reconstitution of severely damaged sciatic nerve using murine skeletal muscle-derived multipotent stem cells. *PLoS One* **9,** e91257 (2014).

211. Giannoudis, P. V, Hildebrand, F. & Pape, H. C. Inflammatory serum markers in patients with multiple trauma: can they predict outcome? *J Bone Jt. Surg Br* **86-B,** 313–323 (2004).

212. Podor, T. J., Jirik, F. R., Loskutoff, D. J., Carson, D. A. & Lotz, M. Human endothelial cells produce IL-6. Lack of responses to exogenous IL-6. *Ann N Y Acad Sci* **557,** 374–377 (1989).

213. Lacey, D. C., Simmons, P. J., Graves, S. E. & Hamilton, J. A. Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation. *Osteoarthr. Cartil.* **2009,** 7 (2009).

214. Baker, N., Boyette, L. B. & Tuan, R. S. Characterization of bone marrow-derived mesenchymal stem cells in aging. *Bone* **70,** 37–47 (2015).

215. Wagner, W. *et al.* How to track cellular aging of mesenchymal stromal cells? *Aging (Albany. NY).* **2,** 224–230 (2010).

216. Duffy, G. P., Ahsan, T., O'Brien, T., Barry, F. & Nerem, R. M. Bone marrow-derived mesenchymal stem cells promote angiogenic processes in a time- and dose-dependent manner in vitro. *Tissue Eng Part A* **Sep. 15,** 2459–2470 (2009).

217. Forsberg, J. A., Potter, B. K., Polfer, E. M., Safford, S. D. & Elster, E. A. Do inflammatory markers portend heterotopic ossification and wound failure in combat wounds? *Clin. Orthop. Relat. Res.* **472,** 2845–54 (2014).

218. Kan, L. & Kessler, J. a. Evaluation of the cellular origins of heterotopic ossification. *Orthopedics* **37,** 329–40 (2014).

219. Kanaji, N. *et al.* Inflammatory cytokines regulate endothelial cell survival and tissue repair functions via NF-κBsignaling. *J Inflamm Res* **2011,** 127–138 (2011).

220. Nathens, A. B. National Trauma Data Bank Annual Report. (2012).

221. Cernak, I. & Noble-Haeusslein, L. J. Traumatic brain injury: an overview of pathobiology with emphasis on military populations. *J Cereb Blood Flow Metab* **30,** 255–266 (2009).

222. ENG - endoglin. (2015).

223. NT5E - 5'-nucleotidase, ecto (CD73). (2015).

224. Woeller, C. F. *et al.* Thy1 (CD90) controls adipogenesis by regulating activity of the Src family kinase, Fyn. *FASEB J.* fj.14–257121– (2014). doi:10.1096/fj.14-257121

225. Ding, J. *et al.* TNF-alpha and IL-1beta inhibit RUNX2 and collagen expression but increase alkaline phosphatase activity and mineralization in human mesenchymal stem cells. *Life Sci.* **84,** 499–504 (2009).

226. Tögel, F. *et al.* Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am. J. Physiol. Renal Physiol.* **292,** F1626–35 (2007).

227. Hager, S., Lampert, F. M., Orimo, H., Stark, G. B. & Finkenzeller, G. Up-regulation of alkaline phosphatase expression in human primary osteoblasts by cocultivation with primary endothelial cells is mediated by p38 mitogen-activated protein kinase-dependent mRNA stabilization. *Tissue Eng. Part A* **15,** 3437–3447 (2009).

228. Kaigler, D. *et al.* Endothelial cell modulation of bone marrow stromal cell osteogenic potential. *FASEB J.* **19,** 665–7 (2005).

229. Rouwkema, J., Westerweel, P. E., de Boer, J., Verhaar, M. C. & van Blitterswijk, C. a. The use of endothelial progenitor cells for prevascularized bone tissue engineering. *Tissue Eng. Part A* **15,** 2015–2027 (2009).

230. Ronchetti, I., Boraldi, F., Annovi, G., Cianciulli, P. & Quaglino, D. Fibroblast involvement in soft connective tissue calcification. *Front. Genet.* **4,** 1–16 (2013).

231. Mako, V. *et al.* Proinflammatory activation pattern of human umbilical vein endothelial cells induced by IL-1beta, TNF-alpha, and LPS. *Cytom. A* **77,** 962–970 (2010).

232. Fairfax, B. P. & Knight, J. C. Genetics of gene expression in immunity to infection. *Curr. Opin. Immunol.* **30,** 63–71 (2014).

233. Wuyts, A. *et al.* The CXC chemokine GCP-2/CXCL6 is predominantly induced in mesenchymal cells by interleukin-1beta and is down-regulated by interferon-gamma: comparison with interleukin-8/CXCL8. *Lab. Invest.* **83,** 23–34 (2003).

234. James, A. W. Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. *Scientifica (Cairo).* **2013,** 684736 (2013).

235. Cenni, E. ANGIOGENESIS AND BONE REGENERATION. *J. Bone Jt. Surgery, Br. Vol.* **87-B,** 58 (2005).

236. Luu, N. T. *et al.* Crosstalk between mesenchymal stem cells and endothelial cells leads to downregulation of cytokine-induced leukocyte recruitment. *Stem Cells* **31,** 2690–702 (2013).

237. Seebach, C., Henrich, D., Tewksbury, R., Wilhelm, K. & Marzi, I. Number and proliferative capacity of human mesenchymal stem cells are modulated positively in multiple trauma patients and negatively in atrophic nonunions. *Calcif. Tissue Int.* **80,** 294–300 (2007).

238. Downey, J. *et al.* Prospective heterotopic ossification progenitors in adult human skeletal muscle. *Bone* **71,** 164–70 (2015).

239. Guihard, P. *et al.* Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling. *Stem Cells* **30,** 762–772 (2012).

240. Seebach, E., Freischmidt, H., Holschbach, J., Fellenberg, J. & Richter, W. Mesenchymal stroma cells trigger early attraction of M1 macrophages and endothelial cells into fibrin hydrogels, stimulating long bone healing without long-term engraftment. *Acta Biomater.* **10,** 4730–41 (2014).

241. Jeon, W. B., Park, B. H., Choi, S.-K., Lee, K.-M. & Park, J.-K. Functional enhancement of neuronal cell behaviors and differentiation by elastin-mimetic recombinant protein presenting Arg-Gly-Asp peptides. *BMC Biotechnol.* **12,** 61 (2012).

242. Ando, Y. *et al.* Stem cell-conditioned medium accelerates distraction osteogenesis through multiple regenerative mechanisms. *Bone* **61,** 82–90 (2014).

243. Ilmer, M., Vykoukal, J., Recio Boiles, A., Coleman, M. & Alt, E. Two sides of the same coin: stem cells in cancer and regenerative medicine. *FASEB J.* **28,** 2748–61 (2014).

244. Lin, H., Yang, G., Tan, J. & Tuan, R. S. Influence of decellularized matrix derived from human mesenchymal stem cells on their proliferation, migration and multi-lineage differentiation potential. *Biomaterials* **33,** 4480–4489 (2012).